An investigation into the detection and identification of OsHV-1 µvar virus and associated risk factors causing mortalities in *Crassostrea gigas* in Ireland.

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Declaration

I hereby declare that the results presented are to the best of my knowledge correct, and that this thesis represents my own original work, carried out during the designated research project period, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work. As this thesis was part of a larger research project, see Appendix 3, other authors made contributions to the published work.

Signed: _____ Date: _____

Acronyms

BIM – Bord Iascaigh Mhara

BLAST - Basic Local Alignment Search Tool

CEFAS – Centre for Environment, Fisheries & Aquaculture Science

Ct – Cycle Threshold

CTD – Conductivity Temperature Depth

CVERA – Centre for Veterinary Epidemiology and Risk Analysis

DNA – Deoxyribonucleic Acid

EFSA – European Food Safety Authority

EPC – Extraction Process Control

EU – European Union

FAO – Food and Agriculture Organization of the United Nations

FFPE – Formalin Fixed Paraffin Embedded

FHU – Fish Health Unit

GMIT – Galway Mayo Institute of Technology

IFREMER – French Research Institute for Exploitation of the Sea

IPC – Internal Process Control

LNA – Locked Nucleic Acid

MI – Marine Institute

NCBI – National Centre for Biotechnology Information

NRL – National Reference Laboratory

OsHV-1 – Oyster Herpes Virus – 1

OsHV-1 µvar – Oyster Herpes Virus micro-variant

PCR – Polymerase Chain Reaction

SQL – Structured Query Language

UCC – University College Cork

UCD – University College Dublin

UK – United Kingdom

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Abstract

The Oyster Herpes Virus micro variant, OsHV-1µvar, is an emerging disease affecting *Crassostrea gigas* oysters which has caused significant mortalities in France, Ireland, and the UK (Jersey and Kent, England) between the summer of 2008 and 2012. The disease is thought to have spread to Ireland with imports of French seed, causing mortalities in oyster stocks of up to 100% in some production areas. In 2008, reports of mortalities in Pacific oysters (*C. gigas*) were received from 3 oyster producing bays in the Republic of Ireland. The presence of the OsHV-1 µvar strain was subsequently confirmed in all 3 bays by PCR and sequence analysis of the amplicon. Extensive mortalities associated with the presence of OsHV-1 µvar were reported from 15 oyster production areas in 2009. This increased to 29 bays with the presence of OsHV-1 µvar confirmed in 2012.

The present MSc. study was carried out in 2 parts.

The first part of the study aimed to characterize and further elucidate the strains of OsHV-1 present in *C. gigas* oysters sampled in Ireland by further examining sequence data obtained from animals collected from 30 bays during mortality outbreaks between 2003-2012.

A retrospective study was conducted on archive formalin fixed paraffin embedded *C. gigas* material from samples associated with mortality outbreaks prior to 2008, to investigate OsHV-1 virus prevalence prior to the reported OsHV-1 µvar outbreak in Ireland in 2008.

Only one OsHV-1 reference strain sequence was identified in one oyster producing bay in Ireland in 2009. Retrospective analysis of formalin fixed paraffin embedded *C. gigas* material, did not find any evidence of the OsHV-1 reference strain in samples collected prior to the OsHV-1 µvar outbreak in 2008. However, one sample, collected in 2007, was found to be positive for the presence of OsHV-1 µvar.

The OsHV-1 µVar strain was shown to be the predominant strain present in Ireland

being detected in all 29 OsHV-1 µvar infected bays, along with another similar genotype of OsHv-1 µVar, with a guanidine insertion and the substitution of a guanine for an adenine residue, which was detected in 6 of the 29 OsHV-1 µvar infected bays.

Part two of this study investigated the detection of OsHV-1 µvar and associated risk factors during mortality outbreaks. An epidemiological study was conducted during 2011. 17 bays from around the Irish Coast were selected, comprising of sites which have been endemically infected by the OsHV-1 µVar virus. Batches of Pacific oysters, *Crassostrea gigas* were selected in each of the 17 bays and were tracked through the study period in an effort to identify any associated management factors. Samples of *C. gigas* oysters were collected from selected stocks in each of the 17 bays to screen for OsHV-1µvar. In addition to pathogen screening and tracking stocks of oysters, environmental monitoring also took place, using sampling points which were located in the vicinity of the point where the samples were collected.

From the analysis of risk factors in 2011, several factors were found significant during mortality outbreaks, including hatchery source, and OsHV-1 viral load in 2011 and the maximum water temperature recorded between June and August 2011.
Multivariable risk analysis indicated that mortalities increased with temperature until a peak was reached.

The current study provides some insights into mortality events affecting farmed Pacific oyster production in Ireland. The epidemiological study conducted found that batch mortality was lower in 2011, compared with earlier years, in association with lower OsHV-1 µvar viral loads.

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Chapter 1 - Introduction

1.1 C. gigas oysters and their role in shellfish production:

The Pacific oyster, *Crassostrea gigas*, was introduced to Europe in the 1960s following a decline in native oyster populations through over fishing and disease, as *C. gigas* proved to be more tolerant to a broader range of environmental conditions and resistant to those diseases (Bonamiosis & Marteliosis) which had affected native oysters. Its rapid growth and ease of cultivation were both contributing factors which makes *C. gigas* one of the most important species of bivalve mollusc reared in the aquaculture industry (Food and Agriculture Organization (FAO)).

The Pacific oyster also plays a significant role in the aquaculture industry in the Republic of Ireland (ROI) both in terms of volume and value, with an annual production of over 7400 metric tonnes in 2011 with a market value of over €27 million (Bord Iascaigh Mhara (BIM) personal communication. 2012). Of all current licensed aquaculture producers in Ireland, oyster farming holds the largest portion of licensed shellfish producers, accounting for over half of all licensed shellfish producers in the country, covering 44 bays all around the coastline.

In Ireland, *C. gigas* seed is predominantly sourced from hatcheries / nurseries or harvested wild seed, which is imported mainly from France and the UK, with the main export market for Pacific oysters being France.

The main method of cultivation for Pacific oysters used in Ireland is bag and trestle cultivation (Figure 1.1), which is an off-bottom culture method. This cultivation method allows the oysters to be placed in the inter-tidal zone which allows access to the stock during low tide (Tidwell , 2012).

Figure 1.1 – Bag & trestle cultivation

Trestles are predominantly metal frame structures, and mesh bags are placed on these trestles and secured down with hooks or rubber bands. The mesh size of the bag is determined by the size of the oyster – spat oysters are generally placed in bags of 4mm size mesh – the purpose of this being to retain the animals within the bag while allowing them sufficient room for growth. The mesh size of the bag prevents the animals from escaping while also being large enough to allow maximum water flow through the bags allowing for feeding and growth. Bags of oysters are then graded and split into larger mesh size bags according to their growth; as the oyster size increases, so too does the mesh size. The production cycle for Pacific oysters in Ireland, from spat to harvest, typically takes 3 years.

Regular maintenance and turning of bags is essential to keep the mesh bags clean and to prevent fouling. Bio fouling of the mesh bags reduces the amount of food available for the oysters to feed on, as many fouling organisms are competitors for the same food supply (Tidwell, 2012).

The main areas in Ireland for oyster production are Donegal Bay, Dungarvan Bay and Woodstown Bay which collectively produce 3650 tonnes of total annual *C. gigas* production, which represents over 50% of the total annual production in the Republic of Ireland.

1.2 Mortality in C. gigas – attributed factors and previous studies:

Summer mortality events in Pacific oysters, *C. gigas*, have been experienced in oyster stocks since the 1950s typically occurring over the warmer months and in adult animals (Samain and McCombie, 2008). The term 'Summer Mortality' describes episodes of abnormal mortality occurring during summer months and mainly affecting Pacific oyster in sexual maturation. The aetiology of summer mortalities in *C. gigas* is multifactorial. Complex interactions between pathogenic agents, environmental factors and genetic and physiological characteristics of the oysters, can be directly or indirectly involved in causing mortalities in *C. gigas* oysters (Samain and McCombie, 2008).

Since the early 1990s, high mortalities have been observed in spat and juveniles of *C. gigas* during the summer period and have been associated with a herpes-like virus in various locations in France (Renault et al., 2000) and similar reports of mortality outbreaks have been investigated in the USA (Burge et al., 2006), Australia and New Zealand (Paul-Pont et al., 2013; Webb et al., 2007).

In the Republic of Ireland, previous studies have been conducted on the investigation of summer mortality in *C. gigas* oysters in the Irish Sea (Malham et al., 2009; Cotter et al., 2010) and these studies relate only to summer mortality events and what factors may be contributing to such mortality events. Cohorts of *C. gigas* oysters were deployed on a number of sites in the Irish Sea to investigate the links between summer mortalities with temperature; nutrients, growth, biochemistry and gametogenesis. The results of these studies on summer mortality in *C. gigas* in the Irish Sea, suggested that a complex interaction between climatic conditions, eutrophic conditions, freshwater input, faster growth and gametogenesis, were the causative factors. Many of these studies focused on specific possible risk factors, however, epidemiological studies are rare across European countries (EFSA, 2010).

Environmental and management factors associated with mortality outbreaks in *C. gigas* oysters will be examined as part of this study, and in addition the relationship between these factors and the prevalence of OsHV-1 µvar in Ireland.

1.3 OsHV-1 virus infection in C. gigas oysters:

Herpes-like viral infections have been identified in various marine bivalve mollusc species, around the world, since the early 1970s (Arzul et al., 2002). Virus detection was often associated with high mortality rates in spat and larvae of farmed bivalves, including the Pacific oyster, *Crassostrea gigas* (Batista et al., 2007) and the European flat oyster, *Ostrea edulis* (Renault et al., 2000). Herpes-like viruses have also been observed in other molluscs such as the Manila clam, *Ruditapes philippinarum* (Renault et al., 2001). The first description of a herpes-like virus morphologically similar to members of the *Herpesviridae* family in bivalve molluscs was presented by (Farley et al., 1972) for the eastern oyster, *Crassostrea virginica*. In 1991, viruses interpreted as belonging to the *Herpesviridae* were associated with high mortality rates of hatchery-reared larval *Crassostrea gigas* in France. Herpes-like viral particles were identified by transmission electron microscopy (Nicolas et al., 1992). This herpes-like virus was later characterised as a member of the Herpesviridae family and called Ostreid herpesvirus 1 (OsHv-1) (Minson et al., 2000).

The economic importance of *C. gigas* and the high level of mortalities observed, particularly in *C. gigas*, lead to the sequencing and characterisation of the Ostreid herpesvirus 1 (OsHV-1) (Davison et al., 2005). Recently, OsHV-1 was included in the re-classification of the Order Herpesvirales as the lone member of the Family *Malacoherpesviridae* (Davison et al., 2009).

Developments in molecular methods, including the use of conventional Polymerase Chain Reaction (cPCR) and quantitative PCR (Q-PCR) to identify OsHV-1, have aided greatly in the study of OsHV-1 infections and permitted the rapid identification and confirmation of OsHV-1 infections in bivalves experiencing high levels of mortalities (Renault et al. 2000, Arzul et al., 2002; Pepin et al., 2008).

In 2000, a variant, of OsHV-1, (OsHV-1var) was associated with mortalities of bivalves

including, *C. gigas*, in French hatcheries (Arzul et al., 2001a). Although this variant genotype presents several modifications in the C region and more than a 2.8 kb deletion, both OsHV-1 and OsHV-1var are considered representative of a single viral species (Arzul et al., 2001b).

Widespread mortalities in *C. gigas* oysters were experienced during the summer of 2008 in France. High levels of mortalities were reported in all production areas and billions of oysters died (Segarra et al., 2010). Mortality outbreaks generally affected spat and juveniles with mortality rates of between 40% and 100% experienced. A study carried out during this time revealed the discovery of another variant genotype, OsHV-1 µvar (Segarra et al., 2010). The characteristics and definition of the OsHV-1 µvar were specified, as a genotype of the virus Ostreid herpesvirus-1 (OsHV-1) which is defined on the basis of the partial sequence data exhibiting a systematic deletion of 12 base pairs in ORF 4 of the genome in comparison with OsHV-1 (GenBank # AY509253) (Figure 1.2). Segarra et al. (2010) also concluded that the polymorphism reported in a microsatellite area in OsHV-1 µVar may be suitable for many applications including strain differentiation and phylogeny studies. This detection of the OsHV-1 µvar genotype and its relationship to extensive mortality outbreaks among Pacific oyster spat suggested an emerging disease situation.

It is now widely accepted that the multifactorial nature of mortality events in *C. gigas* oysters is largely influenced by OsHV-1 µvar infection being necessary but not a sufficient cause. Recent studies have also shown that mortality in *C. gigas* spat can be induced by experimental infection with OsHV-1 µvar and horizontal transmission of infection from unselected asymptomatic adults to juvenile *C. gigas* (Degremont et al., 2013; Schikorski et al., 2011)

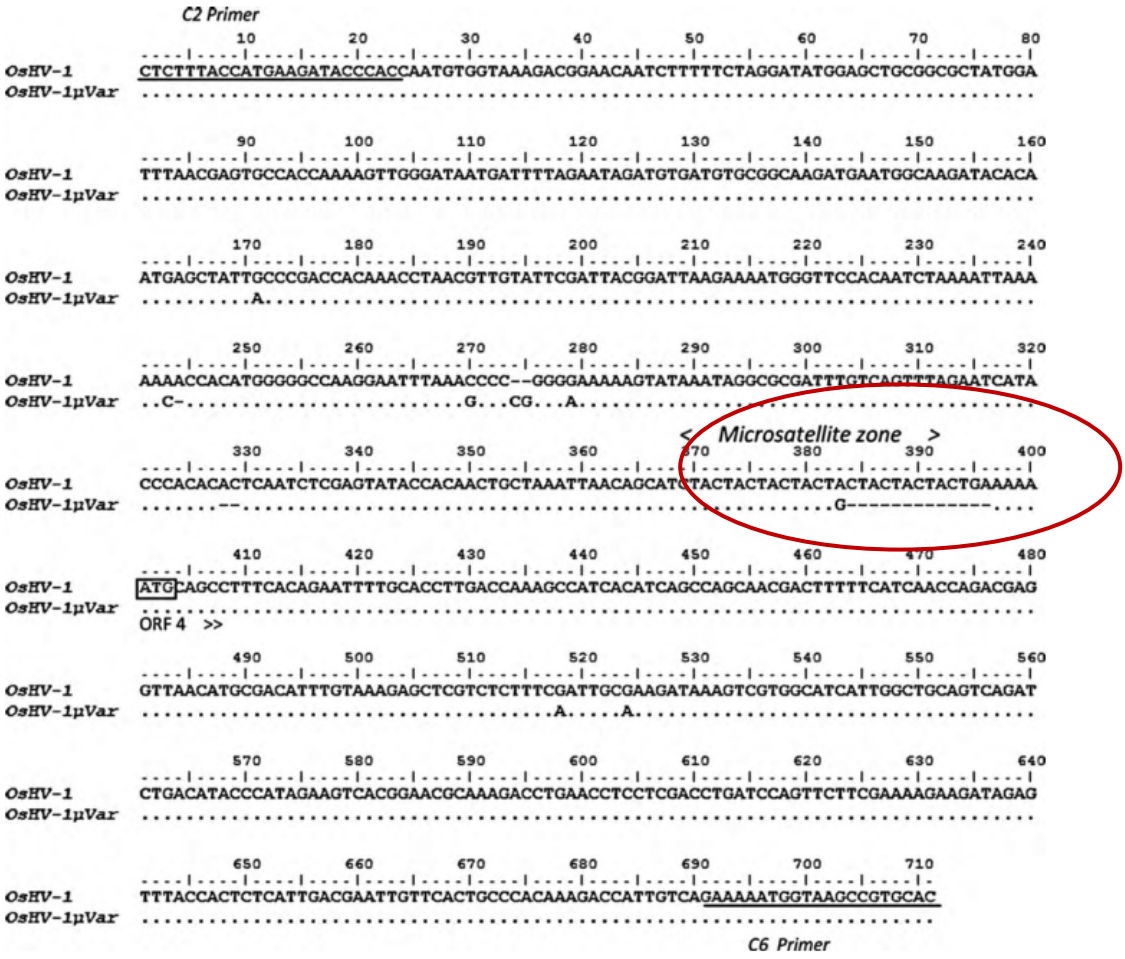

Figure 1.2 – C2/C6 sequence alignments between OsHV-1 consensus and an OsHV-1 µVar isolate. The locations of primers C2 and C6 are underlined. The location of the microsatellite zone before the coding region is indicated (Segarra et al., 2010).

1.4 OsHV-1 variants detected in Europe and Worldwide:

Since 2008, the European aquaculture industry has experienced high mortality rates of up to 80% in *C. gigas*, with significant increases in mortality being reported predominantly in France, the UK and Ireland (Dundon et al., 2011a; Degremont et al., 2013; EFSA, 2010; Peeler et al., 2012; Segarra et al., 2010). This mortality increase in *C. gigas* has been shown to be associated with a new genotype of the Ostreid Herpes virus, referred to as OsHV-1 µvar (Segarra et al., 2010). Many oyster producing areas across Europe have now detected OsHV-1 µvar (Dundon et al., 2011a; Peeler et al., 2012; Roque et al., 2012; Segarra et al., 2010) where the distribution is now widespread

across Europe (Figure 1.3). Previous studies in the United States (Burge et al., 2005) New Zealand (Webb et al., 2007) and Australia (Paul-Pont et al., 2013) have also identified variants of OsHV-1 µvar.

Figure 1.3 - OsHV-1 µvar distribution in Europe – 2012 (image IFREMER, EURL)

The identification and characterisation of OsHV-1 reference strain (Renault et al. 2001a) and the OsHV-1 µvar strain (Segarra et al., 2010) as well as the subsequent discovery of further OsHV-1 strains by Martenot et al., (2012) and Renault et al., (2012), indicated the emergence of further OsHV-1 variants. Recent studies in France have identified new microvariants; OsHV-1 µvar Δ9 which differs from OsHV-1 µvar by a deletion of 9 consecutive nucleotides instead of 12bp deletion in the microsatellite zone, OsHV-1 µvar Δ15 which differs from OsHV-1 µvar by a consecutive deletion of 15 nucleotides in the microsatellite region instead of 12bp deletion (Martenot et al.,

2012).

A study by Renault et al. (2012), analysed clinical OsHV-1 specimens by sequencing amplified fragments from 3 virus genome areas. This study included specimens from France, New Zealand, Ireland, United States, China and Japan. The findings of phylogenetic analysis identified 2 main groups; the first group contained French specimens collected from 1994-2008, samples from the United States and China also were part of this group and the OsHV-1 reference type. The second group contained French specimens collected from 2008-2010, a specimen from Ireland in 2009, and samples from New Zealand and Japan and the OsHV-1 µvar strain was also included in this group. Samples collected in France from 1994-2008 showed some differences from the reference type, which suggests that different OsHV-1 variants existed in France before 2008.

However, the variant OsHV-1 µvar was not detected in French samples collected before 2008 by Renault et al. (2012) which is in accordance with the results presented by Segarra et al. (2010), that OsHV-1 µvar was not detected in archival samples and OsHV-1 µvar was an emerging genotype in Europe.

A recent publication from Ireland reported the first confirmation of OsHV-1 µvar in oyster samples taken before the outbreak reported to the Irish Marine Institute (MI) in 2008, indicating that the emergence of OsHV-1 µvar may have occurred prior to 2008 (Lynch et al., 2012).

The following Chapter 2 will discuss how the OsHV-1 µvar disease situation has developed in Ireland from the initial outbreak in 2008 up to the current situation in 2012. Chapter 2 will focus on an investigation into the infection of OsHV-1 µvar in Ireland, by examining sequence data from OsHV-1 infected oysters taken from all infected bays in Ireland. Chapters 3 & 4 will describe an investigation of the risk factors associated with OsHV-1 µvar related mortality events in *C. gigas*.

1.5 The principal aims & objectives:

The first part of this study (Chapter 1) examined OsHV-1 infection in Ireland, by examining sequence data from selected PCR product data from 30 bays, to establish the variants of the OsHV-1 virus present in Ireland.

- The study aimed to characterize and further elucidate the strains of OsHV-1 present in *C. gigas* oysters sampled in Ireland by further examining sequence data obtained from animals collected during mortality outbreaks between 2003-2012.
- A retrospective study was conducted on archive formalin fixed paraffin embedded *C. gigas* material from samples associated with mortality outbreaks prior to 2008, which aimed to investigate OsHV-1 virus prevalence prior to the reported OsHV-1 µvar outbreak in Ireland in 2008.

The second part of the study (Chapters 3 & 4) aimed to describe mortality events in *C. gigas* oysters in Ireland during 2011 by:

- Identifying any associated husbandry and endogenous oyster factors by examining patterns in management practices in association with mortalities and OsHV-1 µvar virus prevalence (Chapter 3).
- Identifying any significant changes in the environmental parameters in association with mortalities and OsHV-1 µvar infection (Chapter 4).

The overall objective was to provide information which could assist oyster farmers in minimising batch mortality in endemically affected areas.

Chapter 2 – Infection with OsHV-1 µvar- The Irish Perspective.

Abstract –

The genotype OsHV-1µvar has been identified as a new emerging disease in Europe affecting *Crassostrea gigas* oysters which has caused significant mortalities in *C. gigas* oyster stocks in France, Ireland, the UK (Jersey and Kent, England), Spain and Italy along with areas in Australia and New Zealand.

In 2008, 3 Pacific oyster, *Crassostrea gigas* production sites in Ireland reported high levels of mortalities in their stocks. The variant of Ostried herpesvirus-1 (OsHV-1) designated OsHV-1 microvar (µvar) was confirmed in all 3 bays by PCR and sequence analysis. In 2009, 15 bays were confirmed OsHV-1 µvar positive with one OsHV-1 reference strain positive bay and 29 bays were confirmed OsHV-1 µvar positive in 2012.

This study identified the OsHV-1 genotypes present in *C. gigas* producing bays in Ireland by examining sequence data from selected PCR product data from 30 sites, to establish the variants of the OsHV-1 virus present in Ireland. A retrospective study was conducted on archive formalin fixed paraffin embedded (FFPE) *C. gigas* material from samples associated with mortality outbreaks prior to 2008 and found no evidence of the OsHV-1 reference strain prior to 2008. Molecular biology testing using a Locked Nucleic Acid (LNA) real-time PCR method, which is specific for OsHV-1 µvar, found one FFPE sample to be OsHV-1 µvar positive from a cohort of *C. gigas* oysters collected in 2007.

Only one OsHV-1 reference strain sequence was identified in one oyster producing bay in Ireland in 2009. The OsHV-1 µVar strain is the predominant strain present in Ireland being detected in 29 OsHV-1 µvar infected bays, along with another similar genotype of OsHv-1 µVar, with a guanidine insertion and the substitution of a guanine for an adenine residue, which was detected in 6 of the 29 OsHV-1 µvar infected bays.

2.1 Introduction –

2.1.1 Detection of OsHV-1 µvar in Ireland:

In 2008, reports of mortalities in Pacific oysters were received from 3 oyster producing bays in Ireland and the presence of the OsHV-1 µvar strain was confirmed in all 3 bays by PCR and sequence analysis of the amplicon (D. Cheslett, Marine Institute, *pers. comm.*). Extensive mortalities and presence of OsHV-1 µvar were reported from 15 oyster production areas in 2009 (D. Cheslett, Marine Institute, *pers. comm.*). All of these 15 production areas that suffered mortalities, and had the presence of the OsHV-1 µvar strain confirmed in 2009, had imported oysters from France during 2008 or 2009. This evidence suggested that the presence of the OsHV-1 µvar strain was starting to play a significant role in observed mortality events in *C. gigas* in the Republic of Ireland (D. Cheslett, Marine Institute, *pers. comm*.)

In 2009, with numerous mortality events reported and the presence of OsHV-1 µvar confirmed in 15 oyster production areas, a retrospective study was carried out investigating these mortalities in *C. gigas* in order to examine their association with OsHV-1 µvar in the Republic of Ireland (Peeler et al., 2012).

The primary aim of the study by Peeler et al. (2012) was to characterise the pattern of mortality in Pacific oysters in the Republic of Ireland in 2009 in association with OsHV-1 µVar presence and to identify any associated management and environmental factors. A retrospective survey was carried out by issuing an interviewer-administered questionnaire to 70 oyster farmers in an effort to investigate the distribution and determinants of mortality. The results of the study found that there was a strong association ($P < 0.05$) between stock that was introduced from areas that were infected with OsHV-1 µvar i.e. France, with mortality events, when compared with stock that was introduced from areas that the µvar strain of the virus had not been found i.e. UK. This is consistent with disease spread through movements of stock from infected areas. The study revealed that the level of mortality reported varied considerably between bays and between batches of oysters. However, few clear associations with management

factors were identified. The study did, however ,conclude that mortality due to OsHV-1 is likely to be determined by the age of oysters when first infected, the condition of the oysters, temperatures, and other environmental factors (Peeler et al., 2012). The results of the study also suggested that maximum daily immersion and handling were management factors which should be investigated further in future studies. Based on the findings of the study by Peeler et al. (2012), the following chapters will describe the findings of a further epidemiology study that commenced in 2011, which aimed to investigate risk factors associated with OsHV-1 µvar related mortality outbreaks in *C. gigas.*

2.1.2 Regulation to prevent spread of OsHV-1 µvar infection:

In view of the emerging disease situation it became clear that there was a need to control the spread of this OsHV-1 µvar strain through strict regulation of movements of Pacific oysters between production areas across EU Member States. This led to the introduction of the European Union Regulation 175/2010, which was implemented in March 2010. The characteristics and definition of the OsHV-1 µvar were specified in the regulation, as a genotype of the virus Ostreid herpesvirus-1 (OsHV-1) which is defined on the basis of the partial sequence data exhibiting a systematic deletion of 12 base pairs in ORF 4 of the genome in comparison with OsHV-1 (GenBank # AY509253).

As it was thought that the rapid spread of OsHV-1 µvar in France and the Republic of Ireland in 2008 and 2009, may have been attributed to the large-scale movement of Pacific oysters between production areas, European Union Regulation 175/2010 (EU Commission Regulation, 2010) was introduced to restrict the movements of Pacific oysters out of areas that were shown to be infected with OsHV-1 µvar in an effort to prevent the spread of the virus to unaffected areas. This piece of legislation was in force, among all EU member states, from 15th March 2010 to 31st December 2010, whereupon it was extended to the 30th April 2011. EU Regulation 175/2010 introduced the establishment of containment areas, which essentially were areas infected with

OsHV-1 µvar and the establishment of disease free compartments, known as surveillance areas.

A surveillance programme was introduced, which involved a testing regime to ensure the health status of each compartment was known and could be protected. It also laid down restrictions on trade of *C.gigas* between containment areas and surveillance areas. The EU Regulation 175/2010 was then replaced by EU Decision 2011/187 (EU Commission Decision, 2011) on the 1st May 2011, which amended Regulation (EC) No 1251/2008 (EU Commission Regulation, 2008) regarding requirements for placing Pacific oysters on the market in Member States. This Decision retained elements of the previous EU Regulation 175/2010 such as the establishment of disease free compartments with a surveillance programme. It also kept in place restrictions on trade in *C.gigas* into surveillance areas, where all movements of *C.gigas* into surveillance areas must originate from areas which are also free from the disease i.e. other surveillance areas.

Despite having trade restrictions and an active surveillance programme in place, OsHV-1 µvar infection continued to spread to oyster producing bays throughout Ireland and other EU member states, such as the UK during 2010 and 2011, with 29 bays currently positive for OsHV-1 µvar in Ireland in 2012.

2.1.3 Characterisation of OsHV-1 strains associated with mortality events in Ireland:

The discovery of further OsHV-1 strains in France by Martenot et al., (2012) and Renault et al., (2012) has indicated the emergence of further OsHV-1 variants should be investigated in Ireland.

Nucleotide sequence analysis of PCR products from all Irish infected bays reported in EFSA 2010, noted nucleotide variations within the amplicon obtained with C2/C6 primer set. All of the sequences derived from Irish samples taken from infected bays in 2009 were identical to the OsHV-1 µvar sequence except for an insertion of a guanidine

residue in a small number of the samples and the substitution of a guanine for an adenine residue in one of the samples. These sequences were compared to those obtained from the OsHV-1 µvar sequence (GenBank HQ842610) from France. The sequences from samples which did not display the insertion or substitution were identical to the French OsHV-1 µvar sequence (EFSA, 2010).

A recent publication from Ireland (Lynch et al., 2012) reported the presence of OsHV-1 µvar in archive formalin fixed paraffin embedded (FFPE) oyster samples prior to the mortality events of 2008, indicating that the emergence of OsHV-1 µvar may have occurred prior to 2008. Although there had been sporadic mortality outbreaks in *C.gigas* in production sites in Ireland, prior to 2008, the presence of OsHV-1 was never consistently identified.

The current investigation aimed to characterize and further elucidate the strains of OsHV-1 present in *C. gigas* oysters sampled in Ireland by further examining sequence data obtained from animals collected during mortality outbreaks between 2003-2012.
A retrospective study was conducted on archive formalin fixed paraffin embedded *C. gigas* material from samples associated with mortality outbreaks prior to 2008, to investigate OsHV-1 virus prevalence prior to the reported OsHV-1 µvar outbreak in Ireland in 2008.

2.2 Materials & Methods

2.2.1 Samples:

Between 2003-2012 multiple samples of *Crassostrea gigas* oysters were collected from 30 production sites (Figure 2.1) for the purposes of mortality assessment, surveillance programmes and research projects, with the majority of the animals being collected during episodes of mortality and a number of sites being sampled multiple times. Between 2008-2012, live or freshly moribund oysters were sampled and processed for molecular biology testing for the presence of OsHV-1 µvar.

Formalin fixed paraffin embedded (FFPE) *Crassostrea gigas* samples, which were examined as part of this study, were collected between 2003 and 2007 inclusive. In total, 233 formalin fixed paraffin embedded histology blocks were re-examined, representing approximately 328 individual *C. gigas* oysters. These oysters were retrospectively tested using molecular biology techniques for the presence of OsHV-1 µvar.

This testing was carried out in the Fish Health Unit laboratory at the Marine Institute, Oranmore, Co. Galway.

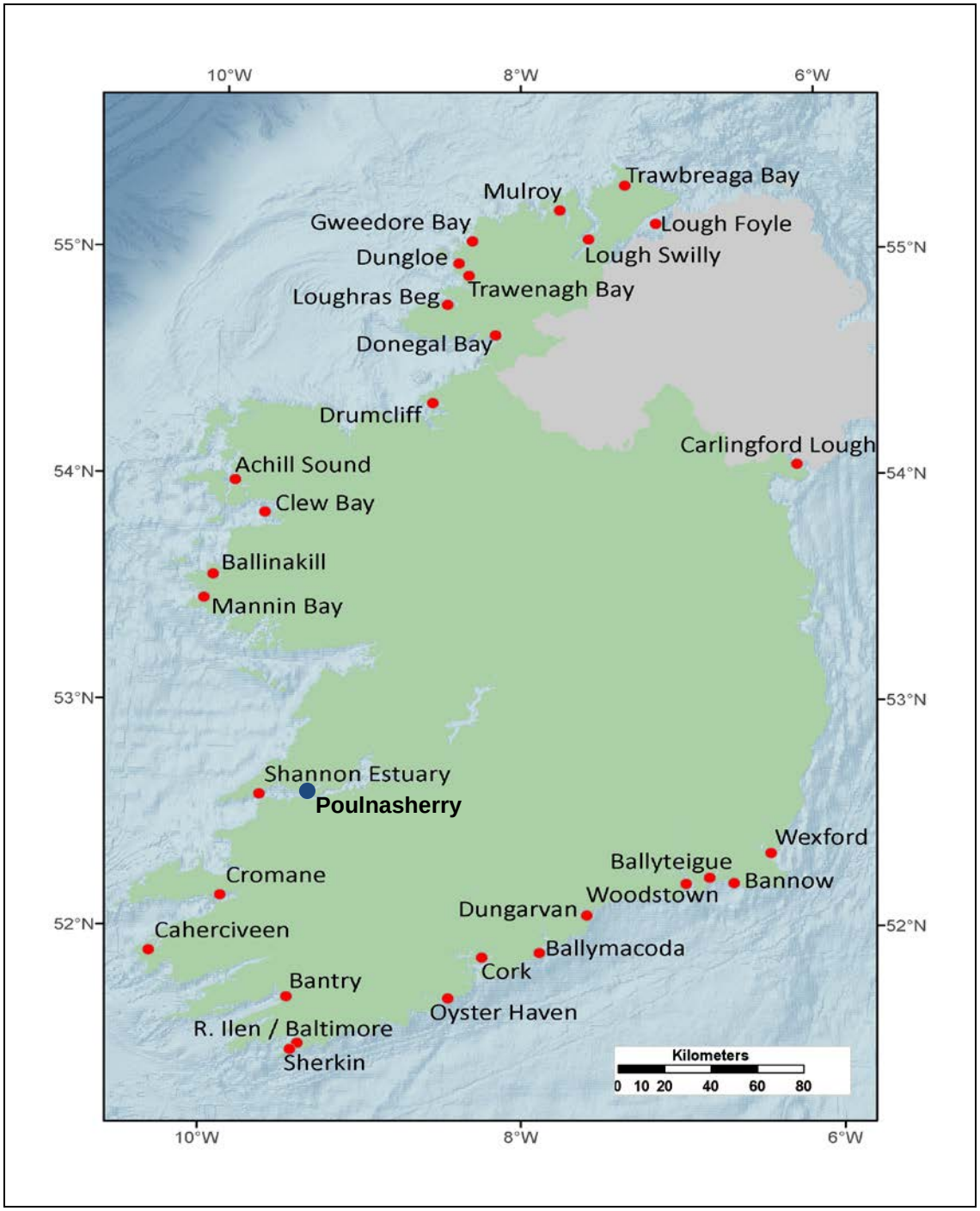

Figure 2.1 – The location of 30 bays included in the study around the coast of Ireland 2012, showing 29 OsHV-1 µvar infected bays (red) and 1 OsHV-1 ref. strain bay (blue)

2.2.2 Sample Preparation:

2.2.2.1 Fresh Oyster Samples: Oyster samples were processed according to their size; For spat smaller than 6 mm, pools of 300 mg of the whole animals (including the shell) completed with 1200 µl of distilled water are crushed and centrifuged at 1 000 g for 1 minute.

For spat between 6 and 15 mm in size, all the soft tissues of each whole animal were crushed individually using Qiagen tissue lyser (Qiagen - TissueLyser II User Manual Sample & Assay Technologies QIAGEN Sample and Assay Technologies, 2010). A ball bearing are placed in each tube and then lysed for 2 minutes at frequency 30 revolutions/second. For animals bigger than 15 mm, pieces of gills and mantle were isolated and were removed using a forceps and scalpel (Figure 2.2). A 2-3mm piece of gill/mantle was removed. Both the forceps and scalpel were wiped with 70% alcohol between dissecting each animal in order to prevent cross contamination.

Sampled tissues, removed from animals as described above, were placed in 2ml Eppendorf tubes, and were either frozen at -20°C for later cell lysis processing or immediately processed for cell lysis and nucleic acid extraction.

Figure 2.2 – *C. gigas* oysters being prepared for dissection

2.2.2.2 Formalin fixed Paraffin Embedded (FFPE) samples:

Deparrafinization of the formalin fixed paraffin embedded (FFPE) specimens was carried out by cutting 8 sections (at a thickness of 10µm for each section) and placing the material in a 2ml eppendorf tube, followed by a xylene wash step with 1ml of

xylene, vortex vigorously for 20-30 seconds and centrifuged at 14000 rpm for 2 minutes (Qiagen Technologies, 2010). This was then followed with an alcohol wash step with 1 ml of 100% ethanol, vortex vigorously for 20-30 seconds and centrifuged at 14000 rpm for 2 minutes. This alcohol wash step was then repeated to ensure that any residual xylene was removed from the tissue. The 2ml eppendorf tubes were then opened and incubated at 30°C for 30 minutes to allow all residual alcohol to evaporate.

2.2.3 Cell lysis and Nucleic Acid Extraction:

All fresh oyster samples and formalin fixed oyster samples included in the study were subject to cell lysis and nucleic acid extraction, which was carried out using QIAamp DNA Mini Kit (Qiagen Technologies, 2007) using Qiagen QIAcube (QIAcube ® User Manual Sample & Assay Technologies, 2008), according to the procedure as described in the manufacturer's instructions. There was an additional incubation step for the paraffin embedded specimens, where after cell lysis all specimens were incubated at 90°C on a thermoshaker for 1 hour, in order to reverse formaldehyde modification and crosslinking of nucleic acids that may have occurred.

2.2.4 Molecular Biology Testing:

Real-time PCR analysis was carried out using Applied Biosystems 7500 Real-time PCR system and all amplification plots were generated and analysed using Applied Biosystems 7500 real-time PCR software.

2.2.4.1 Fresh Oyster sample testing:

A number of different molecular biology diagnostic methods were used throughout the course of this study (Table 2.1). Between 2003-2012, samples were screened for OsHV-1 using various PCR methods including: C2/C6 primer set and PCR conditions described by Arzul et al. (2002); real-time PCR based on a Sybr Green chemistry with C9/C10 primer set targeting the C region of the genome (Pepin et al., 2008); real-time PCR based on Taqman chemistry with B4/BF primers targeting the B region of the

genome (Martenot et al., 2010).

Subsamples of real-time positive PCR products (that gave the strongest signal by amplification by real-time PCR) obtained from samples collected during 2010-2012 were analysed using nested conventional PCR described by David Stone CEFAS, using C2/C6 primers (Arzul et al., 2002) in the first round of conventional PCR, and internal primer set F-int/R-int, with a 514bp expected product size in the second round of conventional PCR, using PCR conditions described by D. Stone, CEFAS (unpublished).

Year	Real-time PCR assay	Conventional PCR assay
2008	N/A	C2/C6 (Arzul et al.,2002)
2009	N/A	C2/C6 (Arzul et al., 2002a)
2010	C9/C10 Sybr Green real-time PCR (Pepin et al., 2008)	C2/C6 & F-int/R-int Nested (Arzul et al., 2002) (D. Stone CEFAS, Unpublished
2011	C9/C10 Sybr Green real-time PCR (Pepin et al., 2008)	C2/C6 & F-int/R-int Nested (Arzul et al., 2002) (D. Stone CEFAS, Unpublished
2012	B4/BF Taqman real-time PCR (Martenot et al., 2010)	C2/C6 & F-int/R-int Nested (Arzul et al., 2002) (D. Stone CEFAS, Unpublished

Table 2.1 - Molecular biology methods used throughout the course of the study

2.2.4.2 FFPE sample testing:

FFPE samples collected between 2003 and 2007, which were selected for this study, were tested for the presence of OsHV-1. Subsamples of extracted DNA from each cohort of FFPE samples were analysed using ThermoScientific Nanodrop 1000, in order to assess DNA recovery and quantify DNA yield.

Archive FFPE specimens were tested in duplicate. Neat DNA extract from FFPE specimens were initially screened using real-time PCR based on Taqman chemistry with B4/BF primers (Martenot et al., 2010). A dilution series was carried out on all FFPE samples to reduce inhibition in the PCR reaction (An and Fleming, 1991; Gilbert

et al., 2007). Samples were diluted by 10^{-1}, 10^{-2} and 10^{-4} to establish which dilution gave optimum results with the least inhibition, and then re-tested using real-time PCR(Martenot et al., 2010).

Subsamples of these FFPE samples (Table 2.2) were tested using a locked nucleic acid (LNA) real-time Taqman PCR method (IFREMER, unpublished), which is specific for the detection of OsHV-1 µvar.

The same subsamples were analysed using nested conventional PCR described by David Stone CEFAS (unpublished).

2.2.5 Process Controls:

Internal Extraction Process Control:

For samples analysed in 2012 and all FFPE samples, an internal extraction process control (EPC) was used to monitor sample extraction efficiency. A real time PCR assay targeting host 18S RNA gene (Biva 18S#19 assay) was run simultaneously with the OsHV-1 assay. The assay, which uses specific primers and probe to amplify a fragment from the host 18S RNA gene, identifies potential false negatives due to inefficient DNA extraction.

External Amplification Control:

An external amplification control assay (Exogenous IPC kit from Applied Biosystems) was also carried out on samples analysed in 2012 and all FFPE samples, to demonstrate differences in PCR efficacy and identify the presence of inhibitors in a sample and hence identify false negatives due to inhibition of amplification.

Positive & Negative Controls

Genomic DNA extracts from *C. gigas* infected with OsHV-1 µvar were used as positive controls, and molecular grade deionised water was used as a negative control.

2.2.6 Sequencing analysis:

Selected DNA products obtained from conventional PCR, with strong amplification, were sent to Sequiserve (Johann-Sebastian-Bach-Str. 785591 Vaterstetten, Germany) for further nucleotide sequence analysis. Individual sequences of all amplified PCR products were aligned using the multiple sequence alignment ClustalW algorithm using MegAlign programme in DNASTAR Lasergene 10 software.

The sequences obtained were aligned with OsHV-1 reference strain (GenBank Accession AY509253), OsHV-1 µvar (GenBank Accession HQ842610) and a variant of OsHV-1 µVar (GenBank Accession JQ963169). Each sequence from amplified PCR products was matched against the National Centre for Biotechnology Information (NCBI) nucleotide database using BLASTn (Basic Local Alignment Search Tool) to evaluate whether the sequences were similar to the OsHV-1 genome, and which variants of OsHV-1 were the closest match to each sequence.

2.3 Results

This study identified the presence of 3 different genotypes of OsHV-1 in oyster producing bays in Ireland.

- OsHV-1 µvar was the predominant strain found to be endemic in 29 oyster producing bays Ireland (2008-2012). There were 2 production sites (Bantry and Ballyteigue) where amplifiable material was not available for sequencing.
- On examination of archive material from formalin fixed paraffin embedded material, the study found one weak positive sample, with a low prevalence, for OsHV-1 µvar in one site in 2007.
- There were isolations of a OsHV-1 µvar sequence with an additional guanidine insertion found in 6 bays (2008-2011).
- OsHV-1 reference strain was identified in one oyster producing bay in 2009. There was no mortality in this bay at the time of sampling.

2.3.1 Molecular Biology testing using Real-time PCR analysis:

Between 2010-2012, samples which were initially screened by real-time PCR analysis, were deemed to be OsHV-1 positive following analysis of fluorescence data**.** All amplification plots were checked to identify inaccuracies such as positive results associated with high background fluorescence and non-sigmoidal amplification. The baseline and threshold of the amplification plots were set so that the threshold crosses halfway up the exponential phase of the amplification plots, when results are displayed in a logarithmic view using Applied Biosystems 7500 software, as shown in Figure 2.3. The number of cycles at which the fluorescence exceeds the threshold is called the threshold cycle (Ct).

Figure 2.3 – Amplification plot analysed using Applied Biosystems 7500 software to show baseline and threshold of the amplification plots with the threshold set halfway up the exponential phase of the amplification plot.

2.3.2 Molecular Biology testing using Conventional PCR analysis:

The characterisation of OsHV-1 genotypes was carried out by conventional PCR analysis in order to amplify DNA products.

In 2008 and 2009, all samples analysed were tested with the C2/C6 primer set (Arzul et al., 2002), as this was the primary diagnostic method used. This methodology amplified DNA products of 709bp.

From 2010-2012, sub-samples of OsHV-1 positive DNA material that gave the strongest signal by real time PCR, were further analysed to amplify DNA products of 710bp with the C2/C6 primer set, and 514bp with the nested PCR using the F-int/R-int primer set (Figure 2.4) in order to obtain PCR products for sequencing.

For the purposes of this study, a total of 88 sequences from 28 oyster production sites were obtained from PCR analysis. Sixty one PCR products were derived using C2/C6 primers with an expected product size of 710bp and twenty seven PCR products were

derived using nested PCR with a primer set of C2/C6 and F-int/R-int primers with an expected product size of 514bp. There are 2 production sites (Bantry and Ballyteigue) where amplifiable material was not available for sequencing. Of these 2 production sites, Bantry bay was confirmed OsHV-1 µvar positive by conventional PCR. Ballyteigue has never been confirmed, however, it is declared positive as it had been in receipt of known OsHV-1 µvar infected stock.

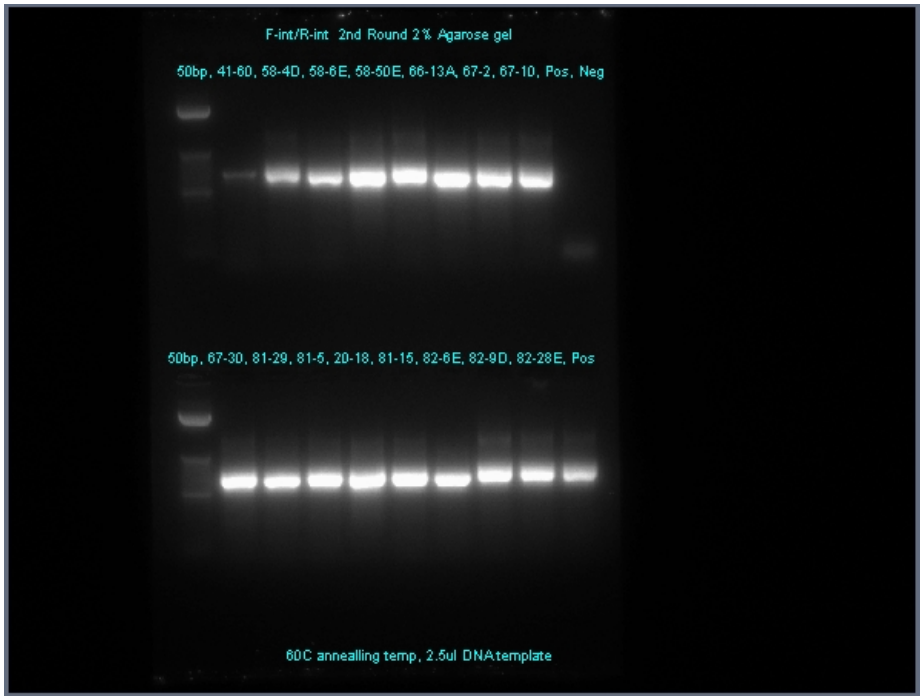

Figure 2.4 – Gel showing amplification of OsHV-1 514bp products using nested PCR using the F-int/R-int primer set.

2.3.3 Formalin fixed paraffin embedded (FFPE) samples:

The results from subsamples analysed using ThermoScientific Nanodrop 1000, showed that DNA recovery was successful and that adequate DNA yield was present to carry out the PCR reaction (Table 2.2)

Results of the dilution series carried out on samples showed that dilutions of DNA extract by 10^{-2} yielded optimum results, with less inhibitors, when compared with the results from neat samples.

The study found one OsHV-1 µvar positive FFPE sample which was collected in 2007.

This FFPE sample was found OsHV-1 positive when initially screened using real-time PCR (Martenot et al., 2010), and was subsequently confirmed OsHV-1 µvar positive by real-time PCR with Locked Nucleic Acid (LNA) probe (IFREMER, unpublished), with a Ct value of 35 (Figure 2.5). This sample was then further tested using nested conventional PCR (David Stone, CEFAS, unpublished). However the limit of detection of this conventional PCR assay is too low to allow amplification of a PCR product suitable for sequence analysis.

The remaining 232 FFPE samples tested negative for OsHV-1.

Figure 2.5 – OsHV-1 µvar from one site in 2007, low prevalence of 1/13 individuals being found positive for OsHV-1 µvar, which was confirmed by real-time PCR with LNA probe (IFREMER, unpublished), with a Ct value of 35

.

Bay/Location	Year Sampled	Number of blocks n=233	Mortality	DNA yield (ng/µl)	Taqman PCR – Martenot 2010	Dilution Series (10^{-2})	Conventional PCR	µvar LNA PCR
Bannow bay	2003-A	19	Yes	251.75 ng/µl	Negative	Negative		
	2003-B	14		227ng/µl	Negative	Negative		
	2003 –C	20	Yes	396.6ng/µl	Negative	Negative		
	2003-D	13		227.6ng/µl	Negative	Negative		
	2003-E	13	Yes	110.2 ng/µl	Negative	Negative		
Castlemaine	2007-A	18	Yes -30%	131.13ng/µl	1/13 Positive	1/13 Positive	slight amplification	1/13 Positive
	2007-B	32	Yes – samples sent in for abnormal morts and OHV testing.	76.15ng/µl	Negative	Negative		
	2007-C	2		84ng/µl	Negative	Negative		
	2007-D	6		43ng/µl	Negative	Negative		
Lough Foyle	2007	31	Yes	311.6ng/µl	Negative	Negative		
Trawbreaga	2007-A1	9	Yes – high morts reported	238.05ng/µl	Negative	Negative		
	2007-B1	6	Yes – 30% morts starting first week august	112.95ng/µl	Negative	Negative		
	2007-C1	12 Formalin 12 Davidsons	Yes – morts in triploids	Formalin – 100ng/µl Davidsons-129.5ng/µl	Negative	Negative		

Table 2.2 – Results of FFPE samples showing OsHV-1 µvar positive sample in 2007 (1/18 *C. gigas* oysters confirmed positive)

2.3.4 Process Controls:

Extraction process control: All sample Ct value results from the Biva 18S#19 assay showed a high degree of homology, indicating that all extraction process results were satisfactory, as the example of results shown in Figure 2.6 displays.

Figure 2.6 – Amplification plot analysed using Applied Biosystems 7500 software showing Biva 18S#19 assay displaying a high degree of homology.

External amplification control (IPC): DNA which was added in addition to sample target DNA to a separate control reaction assay generated a specific result. As such this reaction was used to identify inhibitors present in a sample and identify false negative results due to amplification inhibition; IPC assay results also reported consistent Ct values for each test sample, as the example of results in Figure 2.7 shows.

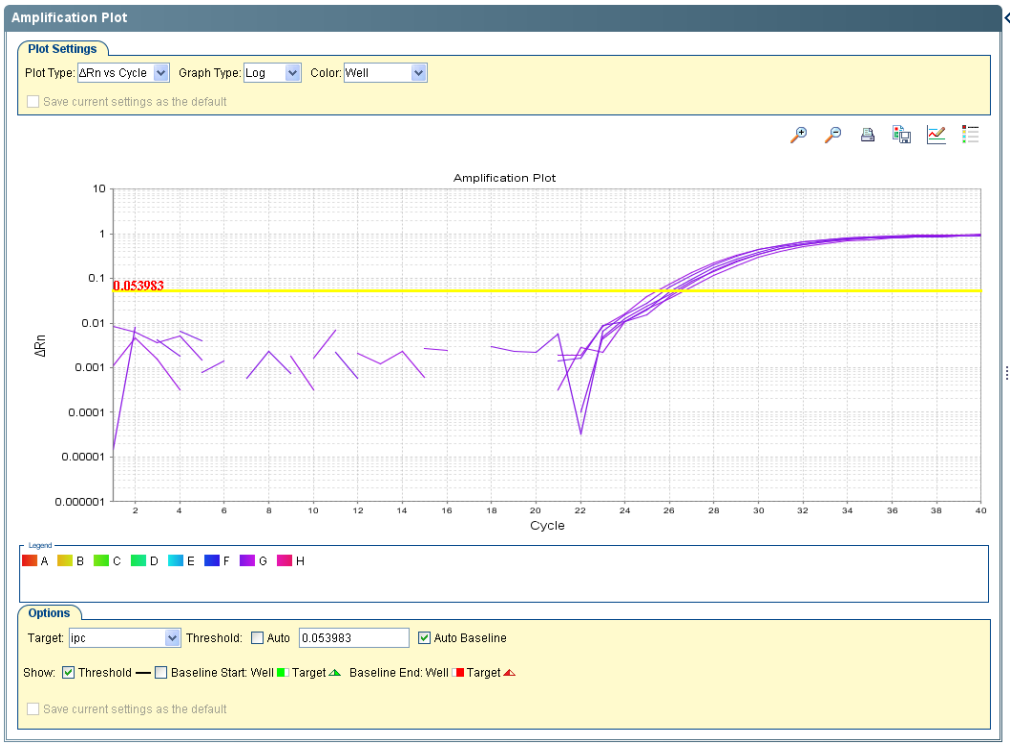

Figure 2.7 – Amplification plot analysed using Applied Biosystems 7500 software showing control DNA and test samples with consistent Ct values.

2.3.5 Sequence analysis:

Sequencing data confirmed that the DNA sequences obtained represented 3 different genotypes of OsHV-1 (Figure 2.8).

At present, 29 oyster producing bays have been confirmed positive for the presence of OsHV-1 µvar around the coast of Ireland (Figure 2.1), from 2008 – 2012.

Sequence analysis of DNA products from 27 of the 29 oyster producing bays around the Irish coastline, were identical to the OsHV-1 µvar sequence (GenBank HQ842610) as described by Segarra et al. 2010.

There are 2 production sites (Bantry and Ballyteigue) where amplifiable material was not available for sequencing. Of these 2 production sites, Bantry bay was confirmed OsHV-1 µvar positive by conventional PCR. Ballyteigue has never been confirmed, however, it is declared positive as it had been in receipt of known OsHV-1 µvar infected stock.

Sequence analysis confirmed that DNA sequences obtained from 6 different oyster producing bays were identical to the OsHV-1 µvar sequence (GenBank JQ963169) an

additional guanidine insertion and the substitution of a guanine for an adenine residue when compared with OsHV-1 µvar (GenBank HQ842610) (Table 2.3).

Only one OsHV-1 reference strain sequence (GenBank AY509253) was identified in one oyster producing bay in 2009.

It was also not possible to carry out sequence analysis on the archive sample from 2007, as described in section 2.3.3 above.

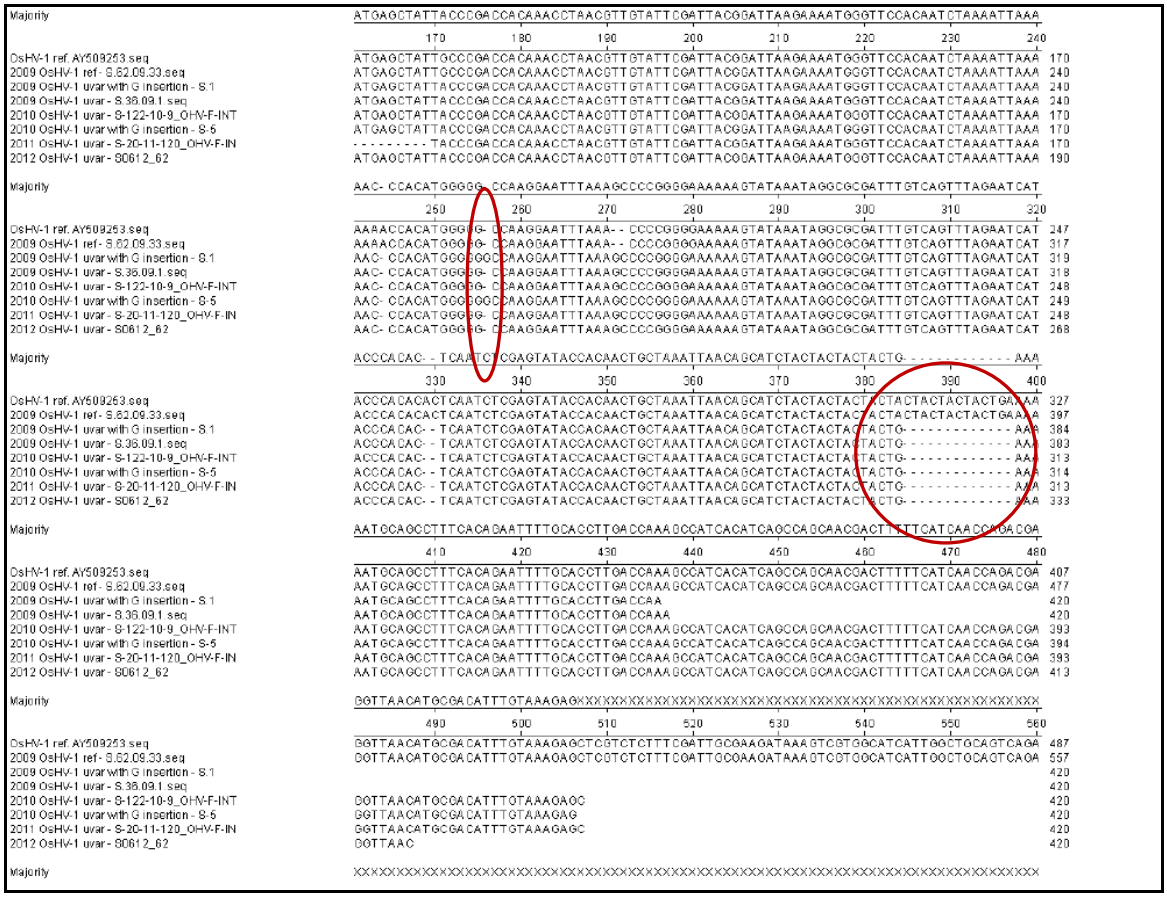

Figure 2.8 – Sequence alignments showing OsHV-1 µvar sequence compared with OsHV-1 reference strain and OsHV-1 µvar with guanidine insertion.

Table 2.3 – Sequences obtained from BLAST sequence analysis

Bay/Location	Year	Mortality Estimate[1] (%)	Number of PCR products sequenced (n=88)	Sequences Identified
1. Woodstown	2008	60-80%	2	OsHV-1 µVar (GenBank JQ963169)
	2009	90%	2	
	2011	40%	1	OsHV-1 µvar (GenBank HQ842610)
	2012	40-50%	4	
2. Castlemaine	2008	30%	1	OsHV-1 µVar (GenBank JQ963169)
	2009	40%	5	OsHV-1 µvar (GenBank HQ842610)
				OsHV-1 µVar (GenBank JQ963169)
	2010	100%	1	OsHV-1 µvar (GenBank HQ842610)
	2011	None	1	
3. Dungarvan	2008	20%	1	OsHV-1 µvar (GenBank HQ842610)
	2009	40%	5	
	2010	40%	4	
4. Carlingford	2009	30%	2	OsHV-1 µvar (GenBank HQ842610)
	2011	50-60%	2	
5. Trawbreaga	2009	65-75%	7	OsHV-1 µvar (GenBank HQ842610)
6. Donegal Bay	2009	40%	3	OsHV-1 µVar (GenBank JQ963169)
				OsHV-1 µvar (GenBank HQ842610)
	2011	40%	2	OsHV-1 µvar (GenBank HQ842610)
	2012	60%	2	
7. Trawenagh	2009	40%	2	OsHV-1 µvar (GenBank HQ842610)
8. Loughras Beag	2009	30%	1	OsHV-1 µvar (GenBank HQ842610)

[1] Mortality Estimate (%) – percentage mortality recorded through farmer recall or estimation of oyster mortality on site at the time of sampling

Bay/Location	Year	Mortality Estimate[1] (%)	Number of PCR products sequenced (n=88)	Sequences Identified
9. Baltimore	2009	40-50%	2	OsHV-1 µVar (GenBank JQ963169)
	2012	None	3	OsHV-1 µvar (GenBank HQ842610)
10. Ballymacoda	2009	40%	3	OsHV-1 µvar (GenBank HQ842610)
	2011	40-50%	1	
11. Dungloe	2009	40%	4	OsHV-1 µvar (GenBank HQ842610)
12. Valentia River/ Caherciveen	2009	40-50%	2	OsHV-1 µvar (GenBank HQ842610)
	2010	15%	1	
13. Achill	2009	60%	2	OsHV-1 µvar (GenBank HQ842610)
	2011	20%	1	
14. Clew Bay	2009	45%	4	OsHV-1 µvar (GenBank HQ842610)
15. Poulnarone Creek	2009	25%	1	OsHV-1 ref. (GenBank AY509253)
16. Lough Foyle	2010	30%	2	OsHV-1 µVar (GenBank JQ963169
17. Mulroy	2010	25%	1	OsHV-1 µVar (GenBank JQ963169)
18. Wexford	2010	None	1	OsHV-1 µvar (GenBank HQ842610)
19. Cork Harbour	2010	50%	1	OsHV-1 µvar (GenBank HQ842610)
20. Bannow bay	2010	None	1	OsHV-1 µvar (GenBank HQ842610)
21. Ballyteigue bay	2010	None	N/A[2]	OsHV-1 µvar
22. Bantry Bay	2010	None	N/A[2]	OsHV-1 µvar
23. Sherkin	2010	50%	1	OsHV-1 µvar (GenBank HQ842610)
	2012	90%	2	
24. Ballinakill	2011	None	1	OsHV-1 µvar (GenBank HQ842610)
25. Gweedore	2011	None	1	OsHV-1 µvar (GenBank HQ842610)
	2012	50%	2	

[2] Amplifiable material was not available from samples collected in these production sites

Bay/Location	Year	Mortality Estimate[1] (%)	Number of PCR products sequenced (n=88)	Sequences Identified
26. Lough Swilly	2009	40%	1	OsHV-1 µvar (GenBank HQ842610)
27. Mannin Bay	2010	20%	1	OsHV-1 µvar (GenBank HQ842610)
28. Shannon Estuary	2012	None	1	OsHV-1 µvar (GenBank HQ842610)
29. Oysterhaven	2012	None	2	OsHV-1 µvar (GenBank HQ842610)
30. Drumcliff	2012	None	1	OsHV-1 µvar (GenBank HQ842610)

2.4 Discussion

On examining sequence data obtained from *C. gigas* samples collected from 30 bays, the OsHV-1 µVar strain was shown to be the predominant strain present in Ireland being detected in 29 OsHV-1 µvar infected bays (Figure 2.1), along with another similar genotype of OsHV-1 µVar, with a guanidine insertion, which was detected in 6 of the 29 OsHV-1 µvar infected bays. Only one OsHV-1 reference strain sequence (Renault and Arzul, 2001) was identified in one oyster producing bay in Ireland in 2009. Retrospective analysis of FFPE *C. gigas* material, did not find any evidence of OsHV-1 reference strain in samples collected before the OsHV-1 µvar outbreak in 2008. Further, one sample, collected in 2007 was found to be positive for the presence of OsHV-1 µvar.

The OsHV-1 µvar strain (GenBank HQ842610) of the virus has been proven to be an emerging disease in Europe in previous studies (Segarra et al., 2010), however, recent studies (Lynch et al., 2012; Martenot et al., 2012; Renault et al., 2012) have found that variants of OsHV-1 have been present prior to the 2008 outbreak, invoking the possibility that the emergence of OsHV-1 µvar was not as sudden as first thought. However, the variant OsHV-1 µvar was not detected in French samples collected before 2008 by Renault et al. (2012) which is in accordance with the results presented by Segarra et al. (2010), that OsHV-1 µvar was not detected in archival samples and that OsHV-1 µvar was an emerging genotype in Europe.

It is widely accepted that OsHV-1 µvar has been the predominant herpes virus strain during mortality events in *C. gigas* since 2009 (EFSA, 2010).

The current study found that isolations of OsHV-1 µvar genotype with additional guanidine insertion and the substitution of a guanine for an adenine residue (GenBank JQ936169) were identified in 6 bays, between 2008-2010. There is no evidence of this genotype of OsHV-1 µvar with an additional guanidine insertion detected in any oyster samples collected after 2010. It was also noted that this OsHV-1 µvar with an additional

guanidine is not exclusive to the particular bays in which it has been found, as both OsHV-1 µvar strains were found in the same oyster production area simultaneously (Table 2.3). Differences in these relatively small nucleotide sequences (when compared to the entire genome) are not sufficient to consider that these are different viruses; they should be regarded as strains of OsHV-1 unless sequence comparisons of more areas of the genome suggest otherwise (EFSA, 2010). The latter would suggest that further investigation is required to examine other regions of OsHV-1 µvar genome in order to establish whether there are additional sequence differences between the isolates.

As part of the present investigation, OsHV-1 reference strain (GenBank AY509253) was identified in one oyster producing bay in 2009, at a very low prevalence of only 1/150 in a cohort oysters found positive for OsHV-1 reference strain. However, this finding was not associated with mortality at the time of sampling, but there was mortality reported weeks prior to the sample being taken, however, the sampling date did not coincide with the mortality event. Although there have been reports of increased mortality in *C.gigas* in the past, OsHV-1 presence was never consistently investigated. There were reports of mortality outbreaks in 1995-1996 in a number of bays, where subsequently samples were sent to IFREMER, France from one of these sites and the OsHV-1 reference strain was detected (EFSA, 2010). In the past, prior to the OsHV-1 µvar outbreak in 2008, mortality reports in *C. gigas* received by the National Reference Laboratory (NRL) for shellfish diseases in Ireland had been relatively infrequent. Testing for the presence of OsHV-1 was carried out in most of these cases of mortality outbreaks, but the virus was never detected.

To further elucidate what strains of OsHV-1 could be present in Ireland; the presence of the OsHV-1 genome in Ireland was investigated during this study by examining archive FFPE oyster samples that were collected during mortality events prior to 2008. There was no further evidence of OsHV-1 reference strain prior to the 2008 outbreak of OsHV-1 µvar, so in this vein it appears that where OsHV-1 reference strain has been detected in the past, it has only ever been detected at a low prevalence in Ireland. This could be attributed to the fact that disease expression of OsHV-1 reference strain has

higher temperature threshold (22°C to 25°C) (Burge et al., 2007; Friedman et al., 2005) than that of OsHV-1 µvar (16°C) (EFSA, 2010), an increase in water temperature which bays in Ireland would rarely reach consistently.

Examination of this archive FFPE material, found one OsHV-1 µvar positive FFPE sample in cohort of 13 samples which were collected during a mortality outbreak in 2007. Mortality rates of 30-40% were reported at the time of sampling. However, investigations carried out at the time by the MI, found that the mortalities appeared to be consistent with summer mortality syndrome due to the timing of the mortalities and the age of the oyster stock affected, as most of the oysters affected were 1 year or older, and all stocks of oysters grown in this bay were originally sourced from France. (D. Cheslett, MI, *pers. comm*.) All stocks sourced from during this time period (2008-2009) may have been exposed to the emerging OsHV-1 µvar genotype. Latent infection could be a factor in the OsHV-1 µvar outbreak, as latency is a common feature of herpes virus infections (Arzul et al., 2002) and prior exposure to herpes virus can lead to latency (Degremont et al., 2013)

The bay in which OsHV-1 µvar was detected from the 2007 FFPE archive material also tested positive for OsHV-1 µvar in 2008 with significant associated mortalities. Further, OsHV-1 testing was carried out at the time (2007) by University College Cork (UCC) but the results were negative. The low level of detection observed in this study, in the sample from 2007 which tested positive for OsHV-1 µvar, suggests that level of viral DNA present in the sample would have been below the level of detection for the PCR methodology used at the time. In 2008, OsHV-1 µvar was identified by conventional PCR (Arzul et al., 2002) and sequencing and results showed a low prevalence of OsHV-1 µvar.

Since the outbreak of OsHv-1 µvar in 2008, efforts have been made in Ireland to understand the spread of OsHV-1 µvar and reasons for new infection have been investigated (Lynch et al., 2012; Peeler et al., 2012). Reasons for the spread of OsHV-1

µvar infection include imports of infected stock from OsHV-1 µvar infected areas, movement of equipment from possibly OsHV-1 µvar infected areas into non-infected areas and sites that were in close proximity to depuration units in non-infected areas, where those depuration units may have taken in molluscs from OsHV-1 µvar infected areas(Peeler et al., 2012). There is also need for further studies of the occurrence and potential transmission of OsHV-1 µvar virus, particularly in the area of vector species being potential source of OsHV-1 µvar infection.

The cause of mortality events in *C. gigas* spat and larvae is multifactorial, with OsHV-1 infection (with OsHV-1 µvar now predominating) being a necessary, but not sufficient, cause(Clegg et al., 2014; EFSA, 2010; Samain and McCombie, 2008)
The detection of OsHV-1 µvar or related variants have also been found in the absence of mortality events, as shown by previous studies (EFSA, 2010) and also in the case of some surveillance areas being found to be OsHV-1 µvar positive despite there being no active mortality reported on site. This suggests that viral infection is influenced by both the host and environmental factors.

Previous studies have investigated the relationship between the host, the environment and the OsHV-1 pathogen, and have found that a complex multifactorial relationship exists between these elements (Burge et al., 2007; Garcia et al., 2011; EFSA, 2010; Paul-Pont et al., 2013; Peeler et al., 2012; Samain and McCombie, 2008). An epidemiology study conducted in Ireland during 2009 (Peeler et al., 2012) gave some insight into how management practices and a range of risk factors could be manipulated in order to reduce the risk of infection with OsHV-1 µvar and also made suggestions as to what should be deemed important for future studies.

The next chapters of this thesis aimed to examine risk factors associated with mortality events in conjunction with OsHV-1 µvar prevalence; will discuss the findings of a further epidemiology study that commenced in 2011 in Chapters 3 and 4 respectively.

***Chapter 3* - An investigation of risk factors associated with management practices affecting the spatial and temporal distribution of OsHV-1 µvar and associated C. gigas mortalities**

This work was part of a collaborative study between the Fish Health Unit of the Marine Institute, Bord Iascaigh Mhara (BIM), and the Centre for Veterinary Epidemiology and Risk Analysis (CVERA) in UCD, which set out to conduct the National Oyster Epidemiology Study 2011-2012 of the effect of OsHV-1µvar on *C. gigas* seed in Ireland in 2011.

Publication - *(Clegg et al., 2014)*‘ Clegg, T.A., Morrissey, T., Geoghegan, F., Martin, S.W., Lyons, K., Ashe, S., More, S.J., 2014. Risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. Prev. Vet. Med. 113, 257–267

I contributed 60% toward the writng of this publication – I was responsible for carrying out the background study and literature review. I contributed to the Study Design, identifying which bays should be included in the study and why. I was resposible for the data collection, design and maintenance of the database which was used for collating the data. I was also responsible for collecting *C. gigas* oyster samples and carrying out analysis for the detection of OsHV-1 µvar. I assisted our colleagues in UCD with interpreting the results obtained from epidemiological analysis and liased with UCD in preparing the resulting publication (Clegg et al., 2014).

Abstract - The study was carried out in 17 OsHV-1 µvar infected bays around the Irish coastline, with the primary aim of describing mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated management factors, thereby providing information which could possibly assist oyster farmers in minimising batch mortality in endemically affected areas. All study farmers were initially surveyed to gather relevant data on each study batch which was then tracked from placement to first grading. At each visit, the cumulative mortality since the last visit was estimated for each batch by asking the farmer for an estimate of the average mortality for the entire batch. A number of risk factors were included for analysis in the study, and all of the factors investigated were analysed and discussed in terms of their influence and relationship with any relevant mortality events in 2011. Where mortality events occurred, details of such events were recorded, along with testing for the presence of OsHV-1 µvar virus, where possible, to assess OsHV-1 µvar prevalence during active mortality.

Primarily this was an investigation into what management practices affect mortality, and how management factors could be adapted in an effort to reduce the impact of OsHV-1 infection.

The results of the study found that there is a lower median batch mortality in 2011, at 16%, when compared with previous years, 2009 (37%) and 2010 (32%).
From the analysis of risk factors in 2011, several factors were found significant, including hatchery source, and OsHV-1 viral load detected in specific bays during 2011. A range of risk factors relating to farm management were also considered but were not found significant.

3.1 Introduction

3.1.1 Establishment of OsHV-1 µvar in Ireland

In Chapter 2, it was concluded that OsHV-1 µvar is now the predominant strain during mortality events in *C. gigas*, with the presence of OsHV-1 µvar virus established in 29 bays around the coast in Ireland.

Although a variant strain of OsHV-1 µvar with an additional guanidine insertion was identified in 6 bays, differences in these relatively small nucleotide sequences, when compared to the entire genome, are not sufficient to consider that these are different viruses; they should be regarded as strains of OsHV-1 (EFSA, 2010).

It is believed that the cause of mortality events in *C. gigas* spat and larvae is multifactorial, with OsHV-1 infection (with OsHV-1 µvar now predominating) being a necessary, but not sufficient, cause (EFSA, 2010). Other risk factors are believed to include an increase or a sudden change in the temperature; husbandry practices such as introduction of possibly infected spat, and the movement and mixing of populations and age groups (EFSA, 2010).

The epidemiology study conducted in Ireland during 2009 (Peeler et al., 2012) gave some insight into how management practices and a range of risk factors could be manipulated in order to reduce the risk of infection with OsHV-1 µvar and also made suggestions as to what should be deemed important for future studies.

3.1.2 Epidemiology study in Ireland in 2009

A study conducted by CEFAS and the Irish Marine Institute (MI) in 2009 on an investigation of OsHV-1 infection and oyster mortality in the Republic of Ireland, aimed to identify management factors to be manipulated to reduce the impact of OsHV-1 infection in infected stocks (Peeler et al., 2012). A number of management factors were examined as part of the study, including the introduction of equipment onto a site, handling of stock, and exposure time of stock i.e. time in or out of water. The study

established that there were few clear associations between management practices and high levels of mortality, with substantial variation in the level of mortality reported within bays that were confirmed positive for OsHV-1 virus. However, the results of the study did recommend factors which should be investigated further in future studies, such as exposure and shore positioning, age at stocking and handling practices.

Increased risk of disease infection was linked to importing new trestles onto the site; this finding was thought to be due to the fact that the introduction of trestles could be an alternative for another variable which could be linked to the disease.
There was no evidence found that increased frequency of handling increases the risk of disease, however, manual handling was shown to be associated with higher levels of mortality in stock when compared with mechanical handling. However, the higher levels of mortalities detected in batches that were handled more frequently could be attributed to the possibility that it is more likely that mortality would be observed in more frequently handled batches. Further investigation of handling practices was suggested for future studies.

Further investigation was also suggested to determine the significance of exposure times for the shortest period of time oysters are out of the water and the longest period of time oysters are out of the water – in order to determine whether exposure time has any significance to disease infection and in conjunction with this to factor in shore positioning.
There was a strong association between stock that was introduced from areas that were infected with OsHV-1 µvar i.e. France, with mortality events, when compared with stock that was introduced from areas that the µvar strain of the virus has not been found i.e. UK. This is consistent with disease spread through movements of stock from infected areas, the study by Peeler et al., (2009) also found that where mortality did occur, mortality started in recently introduced stock early in the summer and occurred later in established oysters. This observation is consistent with the introduction of an infectious agent with new stock that spread to older established stock (Peeler et al, 2012).

Following the findings presented in the study by Peeler et al. (2012), provided the framework for the main aims of the current investigation described in this chapter:

a) To undertake a prospective longitudinal study to investigate the temporal relations between the prevalence of OsHV-1 µvar infection, introduction of stock and the onset of mortality.
b) In addition the study aimed to follow stocks over time to determine temporal relationships between OsHV-1 µvar infection and the interaction of management and environmental factors.

3.2 Materials & Methods

3.2.1 Study Design:

The aim of the study was to track 80 study batches in 17 bays around the coastline (Figure 3.1), throughout the season, from the date of the first batch immersion (3rd August 2010), when oysters were 2mm in size, through to the date of last batch grading before data analysis, in spring/summer 2012 (12th April 2012), where oysters had been immersed for up to 18 months. All 405 batches of *C. gigas* oysters that were being farmed on production sites in Ireland during 2011 were considered for inclusion in the study. Batch selection of the study batches was conducted in order to include batches from different hatcheries, ploidy status and immersion date within each bay. The ability of the farmer to accurately track relevant batches throughout the year was also taken into account.

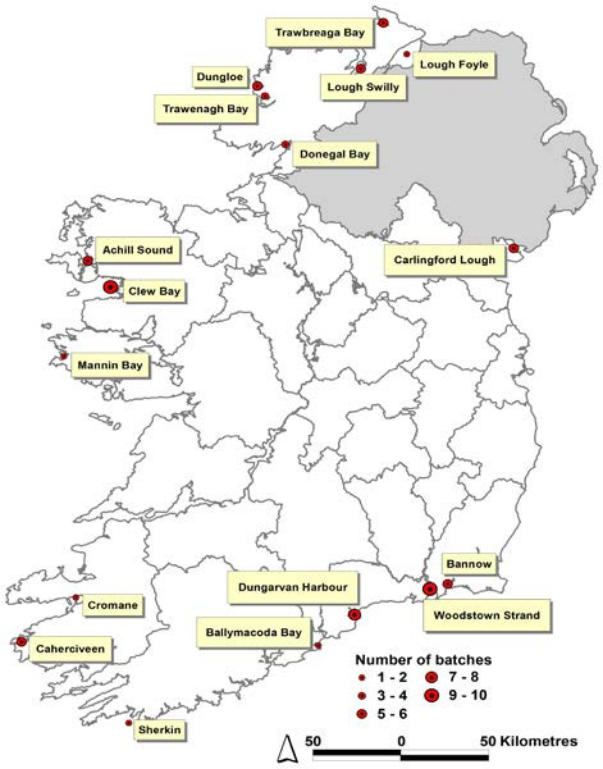

Figure 3.1 – Location of 17 bays containing 80 batches of farmed *C. gigas* oysters involved in the study in 2011

The number of batches required for the study was estimated, based on a confidence interval of 95% (alpha=0.05), a power of 80% and assuming a mortality of 43% amongst batches in OsHV-1 positive bays and 12% in OsHV-1 negative bays (based on reported site-level mortality from the 2009 Irish survey (Peeler et al, 2009). The initial sample size was calculated using a formula to detect a difference between two proportions, assuming other confounders were not strong confounders the sample size was increased by 15% (Doohoo et al., 2009). Clustering of farms was also accounted for by using the following formula: $n' = n (1 + \rho (m -1))$. Where: ρ is the intra-cluster correlation coefficient, assumed to be 0.5 and m is the average number of batches of oysters per farm, assumed to be 3. In total, the aim of the study was to sample around 80 batches.

3.2.2 Data Collation:

A survey was administered jointly by the Fish Health Unit (FHU) of the Marine Institute (MI) and Bord Iascaigh Mhara (BIM) Regional Officers to capture all the data required for the study on each study batch, and to provide a framework for data collection at batch-level throughout the study period. Specifically designed survey forms were administered, with one survey form for each study batch (See Appendix A). Regular visits to the sites were made by the BIM Regional Officers to monitor the progress of each study batch and collect data on batch identification, risk factors of interest, batch mortality data, splitting, grading and handling frequency. For any batch where mortality occurred, details of the mortality event were recorded (i.e. start and finish date of the mortality episode, estimated percentage mortality in the batch, any predisposing factors), and samples of the stock were taken for laboratory analysis where possible. Batch-level data were recorded and compiled in paper format in specifically designed survey forms. All survey forms were submitted to the MI in paper format and were then collated on a specially designed Microsoft SQL server database, which was linked to a Microsoft Access database (Figure 3.2) to allow for manipulation of the data.

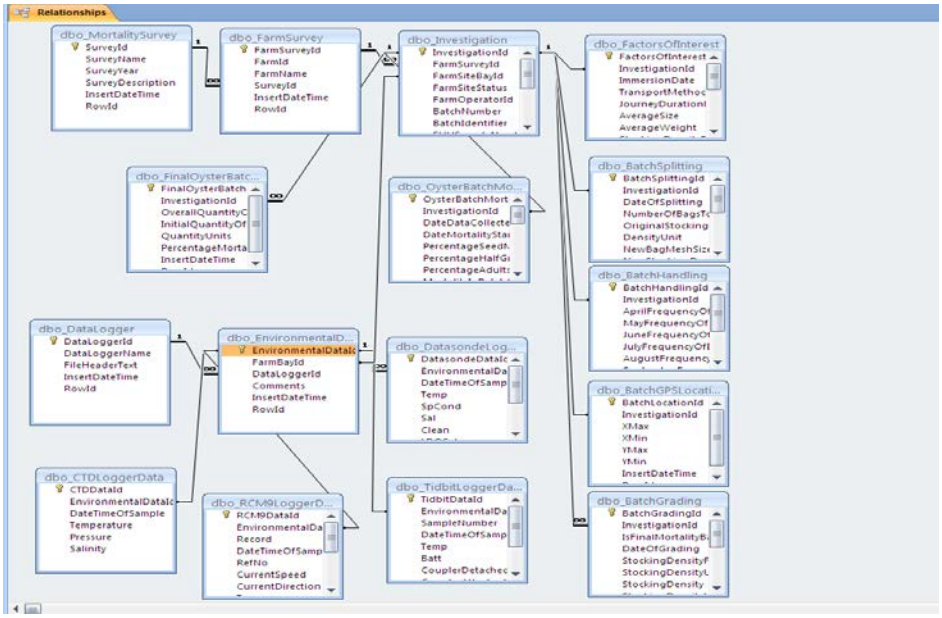

Figure 3.2 – Screen-grab of Microsoft Access database showing relationships between tables of collated data.

3.2.3 Risk Factor Analysis:

The collated data was submitted to the Centre for Veterinary Epidemiology and Risk Analysis (CVERA) in UCD for epidemiological and risk analysis on all the variables considered in the study. The analysis conducted showed that cumulative batch-level mortality (%) from immersion to first grading was the outcome of interest.

A number of independent variables were considered for risk factor analysis (Table 3.1). As the mortality data was highly skewed, the natural logarithm was taken of the mortality data to transform them to a normal distribution. In addition the appropriate transformation of the outcome was assessed based on the residuals from the final model, comparing the likelihood ratio to identify the optimal box-cox transformation (Doohoo et al., 2009) A linear regression model of cumulative batch-level mortality was developed using SAS version 9.1.3 (SAS Institute Inc., 2003). Univariable screening analysis was carried out on all independent variables, with any variables with $p<0.2$ after univariable analysis becoming candidates for multivariable analysis. The need for 'farm' random effect was tested within the mixed model, using likelihood ratio test (Doohoo et al., 2009), to account for clustering of batches within farms. A backward selection procedure was used to eliminate terms from the model based on an F-test ($p>0.05$). Variables that were not significant at the univariable screening stage were added to the final model and tested using an F-test ($p>0.05$). Furthermore, variables were combined to represent an underlying effect and were tested in combination as described by Cohen et al. (2003) by adding the combined variables to the final model. For example, the variables, 'number of times bags were turned' and 'handling at grading' when combined were considered to represent a 'bag handling' effect.

Table 3.1- Independent variables considered in the study

Independent variable	Explanation
Time in water	Measured in days from date of immersion until date of final batch grading. Also grouped into time when placed in water *(2010, Spring 2011 and Summer 2011)*
Age placed in water	*2-3, 4-7, >7 (months)*
Ploidy	*Diploid, triploid*
Bay	17 bays
Position on shore	*Low, mid, high*
OsHV-1 µvar bay status[a]	OsHV-1 µvar detection in 2011 *(positive/negative)*
Bag turning	Number of times each bag turned between May to August 2011
Transport	*Air, sea and road, road*
Journey duration	*≤24 hrs, >24 and <36hrs, ≥36hrs and <42hrs, ≥42 hrs*
Bag mesh size	*<4, 4 and >4 (mm)*
Hatchery	The 7 hatcheries from which the oysters originated
Handling at grading	*Manual, mechanical, both*
Sea bed type	*Gravel, gravel/sand, mud, mud/sand, sand*
Average stocking density	*Average no. of oysters per bag.* If bags were split, oyster density was as estimated at the end of May 2011
Bag split	Whether bags were split during the study into smaller number of oysters *(Yes/No)*
Maximum summer temperature	Maximum temperature reached from the start of June until the end of August 2011, measured at 23 sites with between 1 and 8 batches at each site.

a – Sampling was carried out on a bay basis (i.e. 30 animals from one batch in each bay was sampled for the presence of OsHV-1), however, the batch sampled may not have been part of this study. A bay was considered positive if at least one batch was infected

3.2.4 OsHV-1 uvar Analysis:

OsHV-1 µvar prevalence was assessed by establishing bay status for 2011, which simply established whether a bay was OsHV-1 µvar positive or negative in 2011, also it is important to note here that all 17 bays involved in the study were endemically infected. Sampling was carried out on a bay basis, to assess the prevalence of OsHV-1 µvar in each of the 17 bays in 2011. A sample of 30 oysters was taken from one batch in each of the 17 bays, where the batch sampled may not necessarily be the study batch involved in this study. A bay was considered OsHV-1 µvar positive if at least one batch in the bay was OsHV-1 µvar infected in 2011.

C. gigas oysters were sampled and processed in the same manner as described in chapter two, where all oysters were processed on arrival in the Fish Health Unit laboratory at the Marine Institute as described by EU Regulation 175/2010 Annex I, Part B (States, 2010). Nucleic acid extraction was carried out using QIAamp DNA Mini Kit (Qiagen) using Qiagen QIAcube, according to the procedure as described in the manufacturer's instructions.

DNA extract from the samples was subjected to an initial screening process for the presence of OsHV-1 using real-time PCR based on a Sybr Green chemistry with C9/C10 primer set targeting the C region of the genome,(Pepin et al., 2008).

Confirmatory testing was carried using nested PCR as described in chapter two, where subsamples of real-time positive PCR products were analysed using nested conventional PCR described by David Stone CEFAS, using C2/C6 primers (Arzul et al., 2002) in the first round of conventional PCR, and internal primer set F-int/R-int, with a 514bp expected product size in the second round of conventional PCR, using PCR conditions described by D. Stone, CEFAS (unpublished).

3.3 Results

3.3.1 Risk Factor analysis:

80 batches located on 24 sites in 17 bays were included in the study. Batches contained between 10 and 4,650 bags of oysters, with 215 bags/batch being the average. The number of oysters per bag varied from 210 to 15,000 oysters/bag with 2,000 oysters/bag being the average. Cumulative batch-level mortality varied from 2% - 100% with average batch mortality being 16%, and an interquartile range of between 10% and 34% (Figure 3.3). Therefore, a high mortality site was defined as a site where batch(es) experienced a cumulative batch mortality >34% during the study period. A low mortality site was defined as a site where batches experienced a cumulative batch mortality <34%. 14 high mortality sites (cumulative mortality >34%) were identified with 20 batches in total.

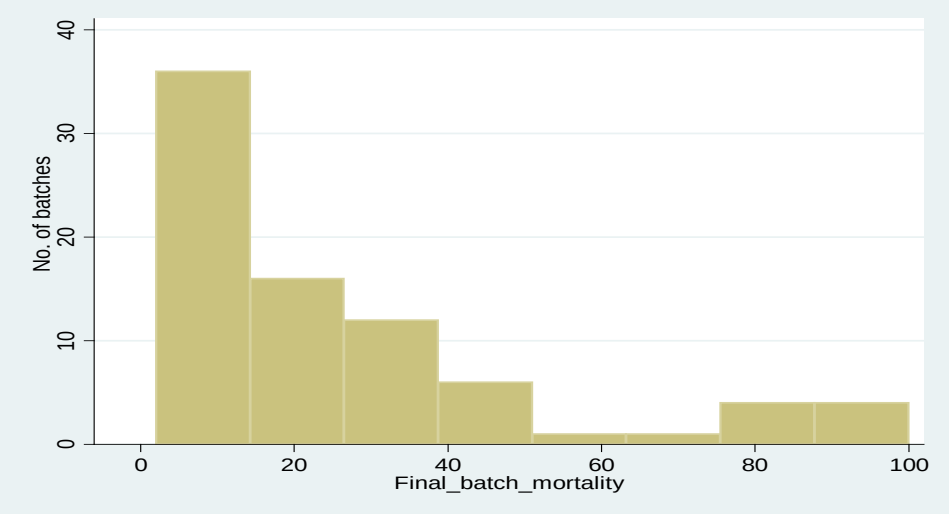

Figure 3.3 – Distribution of the final mortality among the 80 study batches in Ireland during summer 2011

All predictors with a p-value in the univariable analysis of <0.2 were considered in the initial mixed effects regression model. The risk factors entered into the initial model were: ploidy, size, OsHV-1 µvar bay status, and hatchery. The final model after the

backward selection process contained the variables: OsHV-1 µvar bay status and hatchery. Variables that were not significant at the univariable stage ($p>0.2$), and combined variables representing an underlying effect were added to the model, however, none were significant. The natural logarithm transformation was deemed the most appropriate based on residuals and assessment of box-cox transformations and comparing the profile log-likelihood (Doohoo et al., 2009). The 'farm' random effect was significant (likelihood ratio test: $p=0.023$) and 38% of the variance was between farms and 62% within farms.

The model shown in Table 3.2, shows that batches from French hatcheries 1-5 had significantly lower mortalities than batches imported from Non-French hatchery 1 and 2.

Table 3.2 – The median cumulative mortality of oyster batches in Ireland in 2011, along with the interquartile range by categorical risk factors that met the inclusion criteria for the multivariable model (p<0.2)

Risk factor		No. of batches	Cumulative batch mortality			P-value[a]
			Q1	Median	Q3	
Size (mm)	2/3	3	25	39	45	0.083
	4/6	66	9	14	31	
	7 +	11	20	34	57	
Ploidy	Diploid	17	10	33	82	0.059
	Triploid	63	8.7	15	32	
OsHV-1 µvar bay	Negative	13	8	10	12	0.199
status	Positive	67	10	20	35	
Hatchery	French hatchery 1	43	9	13	23	0.005
	French hatchery 2	8	9	19	41	
	French hatchery 3	1	25	25	25	
	French hatchery 4	1	7	7	7	
	French hatchery 5	15	8	15	39	
	Non-French hatchery 1	7	12	86	100	
	Non-French hatchery 2	5	34	76	82	

a. P-value is based on a univariable linear mixed model using log of the final mortality as the outcome variable and 'farm' as a random effect

3.3.2 OsHV-1 µvar Prevalence in 2011:

OsHV-1 µvar status of the bay was significantly associated with mortality in 2011. Further, OsHV-1 µvar negative sites had significantly lower mortalities than positive sites (Table 3.2). In this context, bay status is best interpreted in terms of viral load during 2011, as opposed to presence or absence of OsHV-1 µvar, given that all bays included in the study were OsHV-1 µvar infected.

The viral load of OsHV-1 µvar in each participating bay was assessed during the study and OsHV-1 µvar status was found to be significantly associated with mortality (Figure 3.4). The OsHV-1 µvar results for the Carlingford site show that when mortality occurred, OsHV-1 µvar virus prevalence increased. This finding is consistent with previous work, where it has been shown that there is an increased mortality risk with increasing quantities of OsHV-1 µvar DNA.

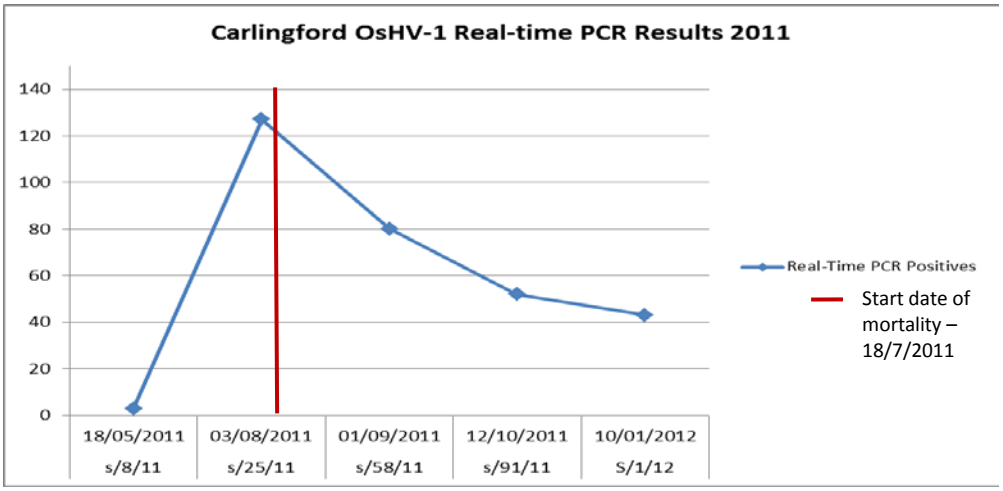

Figure 3.4 – OsHV-1 real-time PCR results for Carlingford Lough during the study period in 2011 showing an increase in virus prevalence associated with active mortality.

3.4 Discussion & Conclusions

The current study showed a lower median batch mortality in 2011 (16%) compared with previous years; 2009 (37%) or 2010 (32%). This current epidemiology study sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. From the analysis of these risk factors in 2011, several factors were found significant during mortality outbreaks, including hatchery source, and OsHV-1 viral load in 2011.

Hatchery Source

There was a strong association between hatchery source and mortality, with French hatchery seed having significantly lower mortality when compared with non-French hatchery seed. There were also differences evident between hatchery stocks, in relation to OsHV-1 µvar exposure. As the OsHV-1 µvar virus is now endemic in France (Pernet et al., 2012) French sourced stock would have been pre-exposed to the virus. As opposed to seed sourced from non-French hatcheries, which are located in areas outside of Ireland and have not been previously exposed to OsHV-1 µvar. This non-French stock would be naive to OsHV-1 µvar, and introduction of naive stock to endemically infected bays appear to have higher mortality rate compared to pre-exposed stock. It has been shown that prior exposure is likely to lead to a degree of latency (Degremont et al., 2013; European Food Safety Authority, 2010), a common feature of other herpesvirus infections (Arzul et al., 2002). Prior exposure will not result in a specific immune response, noting that molluscs lack immunological memory, relying on innate immunity to overcome diseases (Gestal et al., 2008). The resistant effect of prior exposure is likely genetic (Degremont et al., 2007; Huvet et al., 2010; Samain et al., 2007; Sauvage et al., 2009), with oysters surviving a mortality event being naturally selected for resistance to disease (Dégremont et al., 2010; Pernet et al., 2012)

There was a significant univariable association between ploidy and batch mortality with diploid being at greater risk compared to triploid, however, this finding is compromised

by the fact that 88% of French stock was triploid and 75% of non-French stock was diploid. This finding contradicts the with the earlier Irish epidemiology study, where triploid oysters were at greater mortality risk (Peeler et al., 2012). However, the findings of this current study are consistent with studies on adults in France which found triploid oysters were more resistant to summer mortality (Gagnaire et al., 2006; Samain, 2011). It is not possible to disentangle the relative importance of ploidy versus prior exposure factors in the current study, as the batches included in the study were not randomly selected, so there was less variation among batches in terms of ploidy. The observed effect could be due to innate resistance among stock from French hatcheries to OsHV-1 µvar infection.

There was also variation in the mortality of batches from different French hatcheries, with some faring better than others. In these hatcheries, diploid females are produced locally, whereas the male tetraploids are owned by the French government but moved from one hatchery to another for the purposes of fertilization. This may reflect a degree of genetic selection in specific hatcheries over the period since 2007/08 when the disease first appeared in France. A genetic component, leading to hatchery differences, has previously been noted for summer mortality in *C. gigas* adult oysters (Sauvage et al., 2009; Huvet et al., 2010).

OsHV-1 µvar viral load

OsHV-1 µvar viral load was assessed during 2011 using a sampling strategy of 30 *C. gigas* oysters per bay; which provided 95% confidence that viral infection would be detected at a prevalence of 10% or greater if the virus were present. The viral load of OsHV-1 µvar in each participating bay was assessed during the study and OsHV-1 µvar status was found to be significantly associated with mortality, as shown in Figure 3.4. In Carlingford Lough, where samples of *C. gigas* oysters were taken before mortality occurred, during mortality and after the mortality event had ceased. The given start date for the onset of mortality (18/7/11), appears to coincide with an increase in OsHV-1 µvar prevalence (127/150 positive samples with an average Ct value of 27) (Figure 3.4).

This finding is consistent with previous work, where it has been shown that there is an increased mortality risk with increasing quantities of OsHV-1 µvar DNA (Garcia et al., 2011; Pepin et al., 2008; Sauvage et al., 2009; Schikorski et al., 2011). All of the 17 bays included in the study are considered to be endemically infected, with OsHV-1 µvar being detected in each of the bays either during 2011 or in the years previous. The variation of OsHV-1 µvar viral load among different hatchery sourced stock should be investigated in future studies, given the variation in the mortality of batches from different French hatcheries identified by this current investigation. Future studies should aim to investigate the prevalence of OsHV-1 µvar sourced from various overseas hatcheries that would be re-laid in Irish bays under similar husbandry and environmental conditions.

Management Factors

In the current study, a range of risk factors were considered, as outlined in Table 3.1, relating to farm management. This work was guided by international literature and also the findings (and recommendations) from the earlier Irish epidemiology study (Peeler et al., 2012). A range of other management factors were analysed as part of the study such as; Time in water, Bag mesh size, Age placed in water , Journey duration, Handling at grading, Position on shore, Bay, Sea bed type, Bag split, Bag turning, Average stocking density, Transport. All of these farm management risk factors were analysed but were found to be not significant in the current multivariable modelling, despite the role of farm management during mortality events having been emphasized by previous studies (Samain and McCombie, 2008; European Food Safety Authority, 2010). However, the final model only accounted for 24% of the variation in mortality, and therefore there are other risk factors that are not accounted for by the model. Future studies could include other risk factors that were not included in the study, such as investigating the role played by *Vibrio* species during mortality outbreaks. *Vibrio* related species, *Vibrio splendidus*, *Vibrio aestuarianus* and *Vibrio harveyi* have frequently been observed in association with mortality outbreaks prior to and after the 2008 OsHV-1 µvar outbreak. These species have also been detected on oysters not affected by mortality and the role

of *Vibr*io spp. in *C. gigas* oyster mortality is unclear, and should be investigated in further studies (EFSA, 2010).

Conclusions

The primary conclusions of this Chapter are as follows:

- The current study found that there is a lower median batch mortality in 2011, at 16%, when compared with previous years, 2009 (37%) and 2010 (32%).
- From the analysis of risk factors in 2011, several factors were found significant, including hatchery source, and OsHV-1 viral load detected in specific bays during 2011.
- There was a significant association between ploidy and batch mortality with diploid at greater risk, however, this finding is compromised by the fact that 88% of French stock was triploid and 75% Non-French stock was diploid.
- There was also some variation in the mortality of batches from different French hatcheries.

***Chapter 4* - An investigation of key environmental factors affecting the spatial and temporal distribution of OsHV-1 µvar and associated C. gigas mortalities**

This was a collaborative study between the Fish Health Unit of the Marine Institute, Bord Iascaigh Mhara (BIM), and the Centre for Veterinary Epidemiology and Risk Analysis (CVERA) in UCD, which set out to conduct the National Oyster Epidemiology Study 2011-2012 of the effect of OsHV-1µvar on *C. gigas* seed in Ireland in 2011.

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I contributed 60% toward the writng of this publication– I was responsible for carrying out the background study and literature review. I contributed to the Study Design, identifying which bays should be included in the study and why, and also for the deployment and maintenance of loggers when required. I was responsible for the data collection, design and maintenance of the database which was used for collating the data and I assisted in carrying out the visual analyis of environmental data. I assisted our colleagues in UCD with interpreting the results obtained from epidemiological analysis and liased with UCD in preparing the resulting publication (Clegg et al., 2014).

Abstract – this chapter examined the influence any significant change of environmental factors has on the onset of the OsHV-1 µvar virus. In this study, a total of 55 data loggers deployed in 17 bays around the Irish coast in order to obtain environmental data, with at least one data logger deployed per bay. These were deployed in May 2011 on a number of sites, with further deployments made in early June 2011. These data loggers were deployed alongside the trestles which held the batch of interest and were programmed to record every hour for the duration of the deployment, with regular maintenance and calibration on a monthly basis.

The primary aim of this study was to examine the relationship between temperature and the onset of OsHV-1 µvar virus to assess whether the increase in sea water temperature above 16°C threshold coincides with an increase of infectious levels of OsHV-1 µvar viral DNA in *C. gigas* oysters. Furthermore, temperature data was examined to show any patterns in temperature increase or a significant change in temperature levels in conjunction with mortality levels in *C. gigas* oysters.

Results showed that water temperatures were significantly lower in 2011 than in previous years, according to data collected from M1-M5 Databuoys deployed around the Irish coast. Statistical analysis of environmental data showed that unbiased linear estimates of mortality against the 'maximum summer temperature' indicated that mortalities increased with temperature until a temperature peak was reached. Noting that the maximum temperature observed during the study may have occurred as a one day event rather than a sustained rise in temperature.

4.1 Introduction

4.1.1 Environmental factors associated with summer mortalities in C. gigas:

Many previous studies have investigated the influence of environmental factors in association with summer mortality in *C. gigas* oysters. Summer mortality has been associated with multiple stressors such as, phytoplankton blooms, elevated water temperatures, changes in salinity, dissolved oxygen levels and the presence of pathogens. Previous studies have shown that summer mortalities in *C. gigas* cannot be explained by a single factor, but more so by a combination of environmental, genetic, immunological and physiological parameters (EFSA, 2010; Samain and McCombie, 2008). Other suspected risk factors include an increase or a sudden change in temperature, husbandry practices such as introduction of infected spat, and the movement and mixing of populations and age groups (Garcia et al., 2011; EFSA, 2010)

Soletchnik et al., (2007) investigated how environmental factors influence mortality in oysters, by monitoring oyster mortality in one-year old and two-year old oysters at a number of sites along the French coastline over a number of years from 1993-2005, and combining this mortality data with data from environmental monitoring databases. Analyses of both these sets of data showed that environmental factors such as chlorophyll *a* and salinity influence oyster mortality. Chlorophyll *a* concentration, water temperature and turbidity were more significant factors for the mortality of one-year old oysters, while salinity and chlorophyll *a* had more significant effect on two-year old oysters.

Soletchnik et al., (2007) also found that water temperature is significantly linked to one-year old oyster mortality, predominantly in the spring-summer seasons. Summer chlorophyll *a* levels showed a significant correlation with juvenile mortality rate and chlorophyll *a* levels of the autumn-winter period was significantly linked to mortality of two-year olds oysters. Although Soletchnik et al., (2007) showed links between the influence environmental factors and oyster mortality, the study was not specific to *C.*

gigas species but gave insight for further investigation into additional stress parameters which could be causative to mortality events.

4.1.2 Environmental factors associated with OsHV-1 infection:

In a study in Tomales Bay in the USA, Burge et al., (2006) examined the roles of environmental factors, the host and pathogens in the host-pathogen relationship between OsHV-1 and *C. gigas.* Significant trends were observed in oyster mortality patterns which significantly correlated positively with maximum temperatures. However, Burge et al., (2006) found that elevated temperature was the only environmental factor consistently related to mortalities, as extreme losses occurred when temperatures exceeded 24-25°C. For example, in 2003, elevated temperature means predicted OsHV-1 presence and similarly OsHV-1 presence predicted mortalities. Data from both the field and lab experiments indicated an infectious disease, such as, OsHV-1 acts in conjunction with temperature to kill *C. gigas* in Tomales Bay.

OsHV-1 DNA was first detected 2 weeks after the first maximum temperature peak at 25°C and based on this finding it is hypothesized that temperature peak over 25°C may induce OsHV-1 viral replication. Burge et al., (2006) showed that increased thermal exposure of *C. gigas* oysters gave rise to faster viral replication and earlier mortalities, and concluded that OsHV-1 virus and elevated temperatures were the most likely cause of summer mortality in *C. gigas* observed during the study. Burge et al., (2006) concluded that OsHV-1 in conjunction with these 2 factors; mortalities and extreme temperatures, are playing a role in summer mortality in *C. gigas* in Tomales Bay, California.

Le Deuff et al., (1996) observed herpes-like viral infections and 100% losses within 6 to 13 days after being exposed to temperature of 25°C. In contrast, transmission of OsHV-1 to larval oysters was unsuccessful at temperatures between 22-23°C.
Furthermore, the European Food Safety Authority (2010) noted that OsHV-1 outbreaks in Europe during 2008 and 2009 occurred when water temperatures were above 16°C. The 2009 epidemiological study by Peeler at al., (2012) suggested that future studies

should aim to follow stocks over time better to determine temporal relationships between infection with OsHV-1 µVar, other risk factors (management or environmental), and mortality. However, under some circumstances, certain environmental factors or conditions may also be required for disease to occur.

The current study aimed to investigate a number of environmental parameters and examine their relationship with *C. gigas* mortalities and the OsHV-1 µvar virus in 17 bays around the Irish coastline.

4.2 Materials & Methods

4.2.1 Study Design and Data Loggers; Deployment & Maintenance:

The study took place during the summer of 2011 in conjunction with risk factor analysis of management factors (Chapter 3) and also focused on the same 17 bays round the coastline as described in Chapter 3 (Figure 3.1).

A total of 55 data loggers, at least one per bay, were deployed to obtain environmental data. These were deployed in May 2011 on a small number of sites, with further deployments made in early June 2011 on the remaining sites. A data logger was placed on each participating site. Five different types of data loggers were deployed (two large types and three small types), each recording a number of different parameters.

Two "large" data loggers included:

- Hydrolab DS5 Multiparameter Datasonde loggers (Figure 4.1 & 4.2): temperature, salinity, conductivity, dissolved oxygen, pH, pressure, chlorophyll.
- RCM9 loggers: temperature, current, salinity, depth, pressure.

Figure 4.1 - Hydrolab DS5 Multiparameter Datasonde

Figure 4.2 – Hydrolab datasonde deployed on site

Three "small" data loggers included:

- Star-Oddi DST CTD loggers (Figure 4.3): temperature, salinity, pressure.
- Star-Oddi Pressure-Temperature loggers: temperature, pressure.
- StowAway TidBit logger (Figure 4.4): temperature.

Figure 4.3 - Star-Oddi DST CTD logger

Figure 4.4 - StowAway TidBit

The smaller data loggers were deployed attached to the bags in the batch of interest, whereas the larger data loggers were deployed alongside the trestles which held the batch of interest (Figure 4.5). These data loggers were programmed to record environmental parameters every hour for the duration of the deployment, with regular maintenance and calibration on a monthly basis.

Hydrolab DS5 Multiparameter Datasonde loggers were deployed in Dungarvan Bay, Donegal Bay, Carlingford Lough and Clew Bay. There were some technical issues with these loggers throughout the sampling season, resulting in some missing data. However, these 4 bays also had small data-loggers on-site, so those data were used as back-up. RCM9 loggers were deployed in Dungarvan and Woodstown bays. Each bay had a Star-Oddi DST CTD logger, and some bays had additional StowAway Tidbit logger if there was more than one site involved in the study within the bay. Star-Oddi Pressure-Temperature loggers were also deployed as additional loggers in bays where there was more than one site involved in the study.

Data were downloaded on monthly basis for datasondes; monthly/6 weeks for CTDs; tidbits were downloaded after a longer deployment period as they were better able to withstand the conditions, had a longer battery life and required very little maintenance.

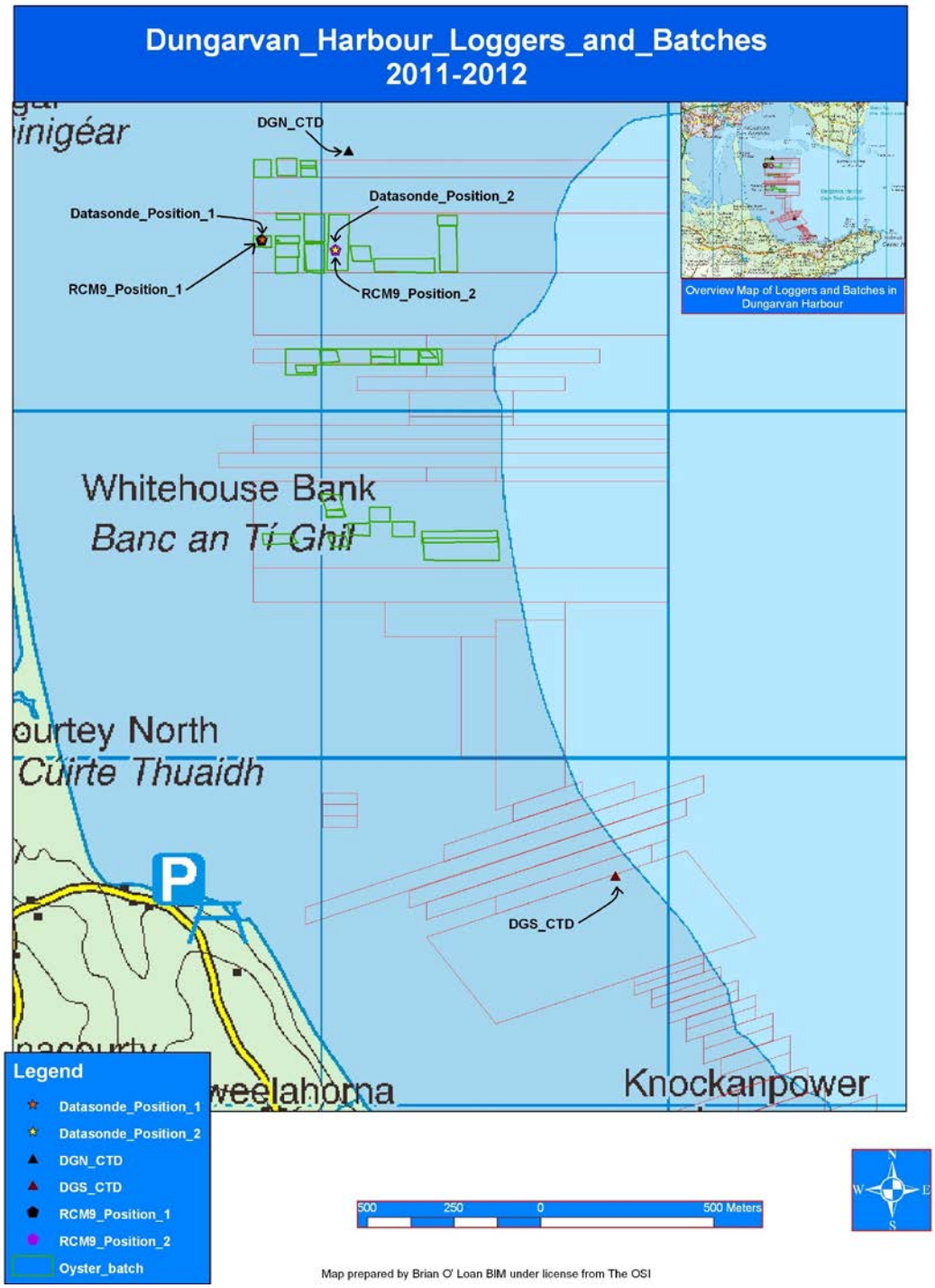

Figure 4.5 – The location of study batches and environmental data loggers in Dungarvan Bay during 2011-12

4.2.2 Data Collation:

Data were downloaded from the data loggers in Microsoft Excel format. Data were collated and organised on a bay basis. Data were imported onto a Microsoft SQL server database which was linked to Microsoft Access database, containing all batch data detailed in Chapter 3, to allow for data manipulation (Figure 4.6).

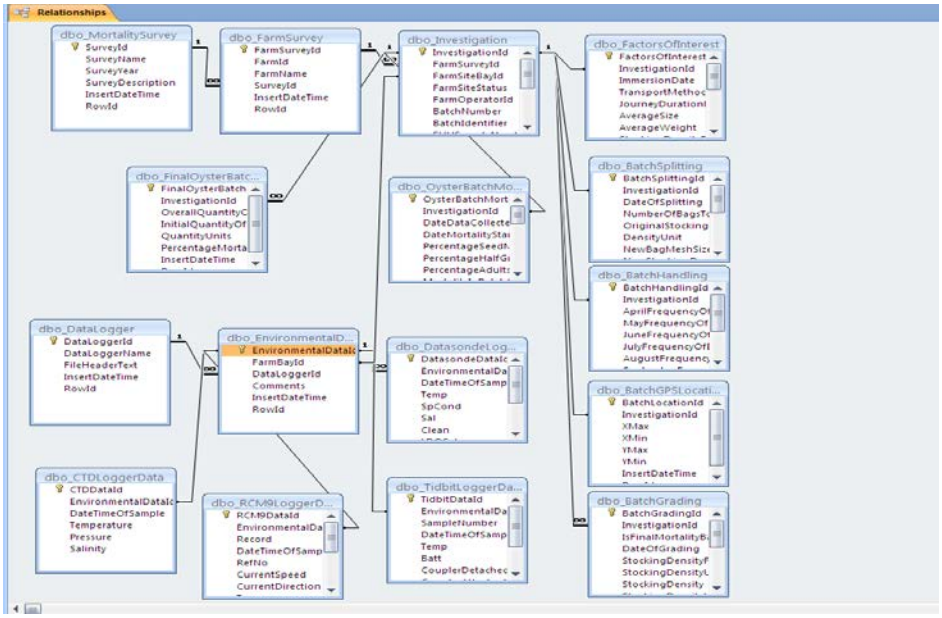

Figure 4.6 – Screen-grab of Microsoft Access database showing relationships between tables of collated data.

4.2.3 Statistical analysis of Environmental data:

The collated environmental data were submitted to the Centre for Veterinary Epidemiology and Risk Analysis (CVERA) in UCD for analysis of all environmental parameters collated in the study. A range of environmental parameters were available for assessment, however, temperature was the only factor deemed significant for further analysis. At all high mortality sites with available data (reported start date of mortality event and temperature data during the 30 preceding days), the median temperature within the previous 7 and 30 days prior to the recorded start of mortality was reported (Figure 4.7). The median temperature at all low mortality sites was also reported, during the same time periods as the high mortality sites. This was to allow for comparison

between low and high mortality sites during the same time period.

As previously stated in Chapter 3, based on the interquartile range of cumulative mortality, a high mortality site was defined as a site where 1 or more batches had cumulative batch-mortality >34%, and a low mortality site was defined as a site where no batches experienced cumulative batch-mortality <34%.

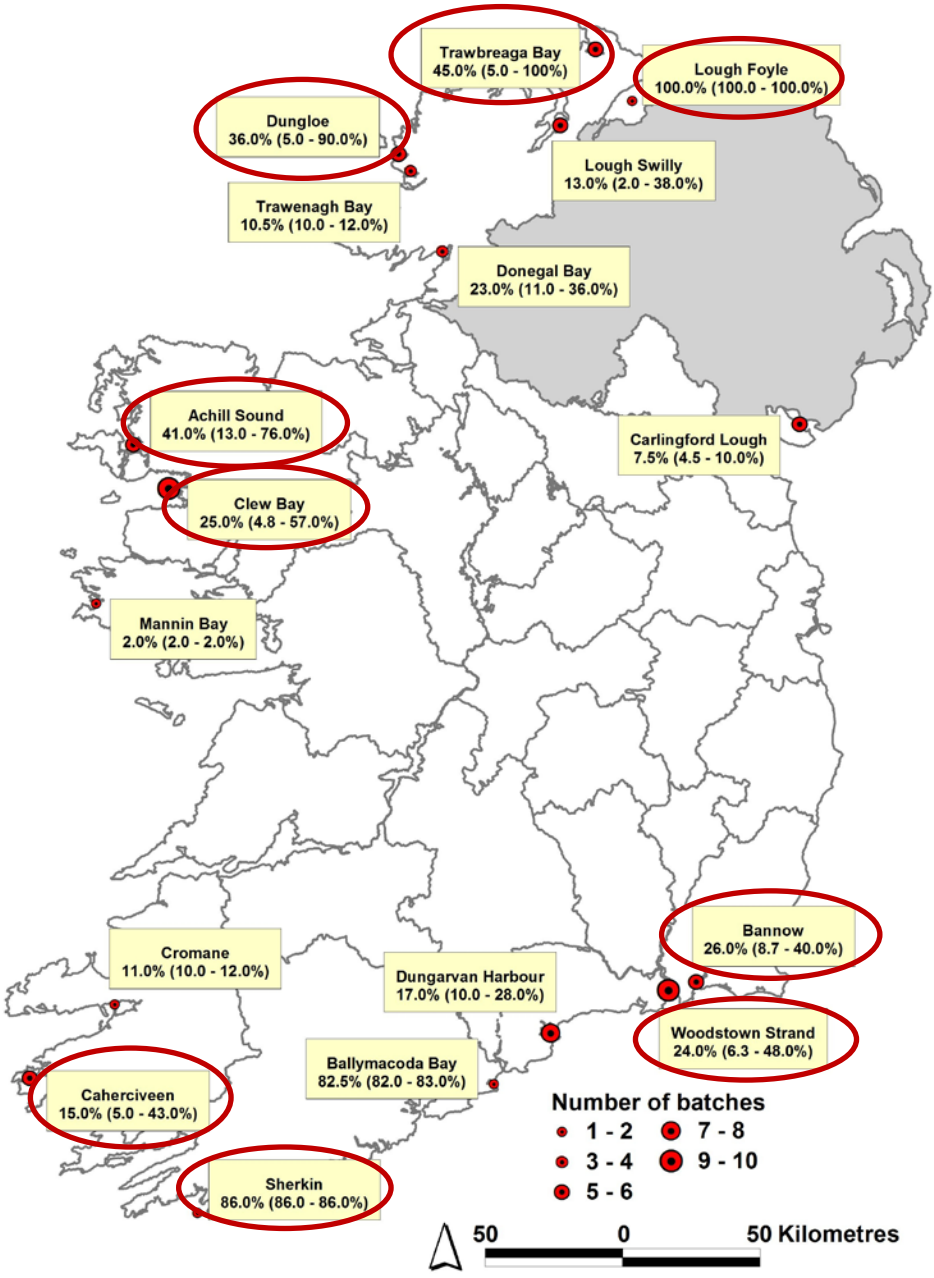

Figure 4.7 – 17 bays included in the study, with the 10 high mortality sites that had recorded start dates for mortality

4.2.4 Visual Assessment:

For seven sites where high mortalities were reported in 2011, a visual assessment was made of temperature changes during the period when mortality events were first recorded. Data were filtered using a low-pass filter, using Matlab (MathWorks, Natick, MA, USA) to show the overall temperature trend without tidal/diurnal variability. This was used to assess the overall trend in the temperature pattern during the time period when mortality occurred, particularly with respect to the 16°C threshold. The filtered temperature data were then graphed using Microsoft Excel 2007 using a scatter plot with smooth lines, coloured lines were used to highlight the 16°C threshold (red) and the reported date for onset of mortality (green) (Appendix B).

4.3 Results

4.3.1 Environmental Data Analysis:

The results from risk factor analysis in Chapter 3 showed that there were 14 high mortality sites, with a total of 20 batches. Of these 14 high mortality sites, 10 sites had a recorded start date for mortalities. At three of these sites, the start of mortality or related temperature data were missing, including Ballymacoda Bay (temperature data were missing within the 2 weeks immediately prior to the recorded start of mortality), Bannow (temperature data were missing prior to the recorded start of mortality), and Lough Foyle (no temperature data were available).

The median temperature within 7 and 30 days of the reported start of mortality and relevant temperature data at 7 high mortality sites (all but Ballymacoda, Bannow and Lough Foyle, with insufficient data) is shown in Table 4.1. The analysis of temperature data found very little difference in temperature between high and low mortality sites prior to the start of mortality, which indicated that temperature was not a significant factor in the cases observed in 2011.

The interquartile range for the high mortality sites overlapped the interquartile range for the low mortality sites during the 7 and 30 days time intervals, with the exception of Lough Swilly, indicating that there was little difference in temperature between the low and high mortality sites prior to the start of mortalities. It was therefore concluded that there was no association between temperature and observed disease outbreaks. However, the final multivariable risk factor model indicated that mortalities increased with temperature until a peak was reached.

Table 4.1. The median and interquartile range of temperature recorded within 7 and 30 days of the reported start of mortalities at seven high mortality sites (those with 1 or more batches with a cumulative mortality > 34%) during summer 2011 in Ireland. The median and interquartile range of temperatures at the ten low mortality sites (those sites without any high mortality batches) during the same time periods is included for comparison.

		At each high mortality site			Among the ten low mortality sites[a]		
		Temperature (°C)			Temperature (°C)		
Bay[a]	Reported start of mortality	Median	interquartile range		Median	interquartile range	
			Q1	Q3		Q1	Q3
Within 7 days of start of mortalities							
Achill Sound	14-Jul-11	15.9	14.9	16.7	15.3	14.5	16.5
Clew Bay	20-Jul-11	14.8	13.9	15.8	15.0	13.7	16.7
Dungloe	03-Jul-11	15.7	15.3	16.9	14.4	13.5	15.7
Lough Swilly	22-Jul-11	18.8	15.5	20.0	14.4	13.4	15.4
Sherkin	11-Aug-11	14.8	14.5	15.1	15.9	15.4	16.3
Trawbreaga Bay	26-Jul-11	15.2	14.4	16.9	14.9	14.1	16.1
Woodstown Strand	26-Jul-11	14.8	14.4	15.6	14.9	14.1	16.1
Within 30 days of start of mortalities							
Achill Sound	14-Jul-11	17.0	15.6	18.8	19.2	18.0	19.8
Clew Bay	20-Jul-11	15.0	14.2	15.8	15.3	14.0	18.5
Dungloe	03-Jul-11	15.5	15.0	16.2	19.3	18.4	19.9

Lough Swilly	22-Jul-11	20.5	17.4	22.9	14.8	13.7	16.0
Sherkin	11-Aug-11	14.5	13.7	14.9	15.8	14.8	16.5
Trawbreaga Bay	26-Jul-11	15.2	14.3	16.3	15.0	14.0	16.3
Woodstown Strand	26-Jul-11	15.1	14.6	15.7	15.0	14.0	16.3

a. The bay may also contain other sites

4.3.2 Visual Assessment:

A visual assessment of temperature data during the reported start of mortality at 7 high mortality sites (all but Ballymacoda Bay, Bannow and Lough Foyle, with insufficient data) is presented in Appendix B. At four sites (Achill Sound, Dungloe, Trawbreaga Bay and Woodstown Strand), an increase in temperature over 16°C coincided with the time period when mortality events were first reported; at one site (Sherkin), no visual association was observed; and at two sites (Clew Bay, Lough Swilly), a substantial change in temperature, in this case a decline followed by an increase, was coincident with the time period when mortality was first reported.

4.3.3 Temperature and Mortality events:

Water temperatures were significantly lower in 2011 than in previous years, according to data collected from M1-M5 Databuoys deployed around the Irish coast, (Figure 4.8). a significant decline in average sea water temperature can be observed from the Figure 4.8, where sea water temperature is much lower when compared with that of previous years.

No consistent temporal relationship was identified between water temperature (7 and 30 days prior to the start of mortalities) and subsequent mortality events. In addition, there was very little difference in temperature between the low and high mortality sites prior to the start of mortalities (Table 4.1).

Univariable analysis conducted on variables outlined in Chapter 3, also included maximum summer temperature as a variable in the initial linear mixed regression model. The Loess plot against ‘maximum summer temperature’ suggested a possible quadratic relationship between the natural logarithm of the cumulative mortality and the maximum temperature observed between June and August 2011. It was also noted that the maximum temperature observed during this study may have occurred as a one day event rather than a sustained rise in temperature. A plot of the best unbiased linear estimates of mortality against the ‘maximum summer temperature’ is shown in Figure. 4.9, indicating an increase in mortality which appears to level off after a temperature

peak was reached.

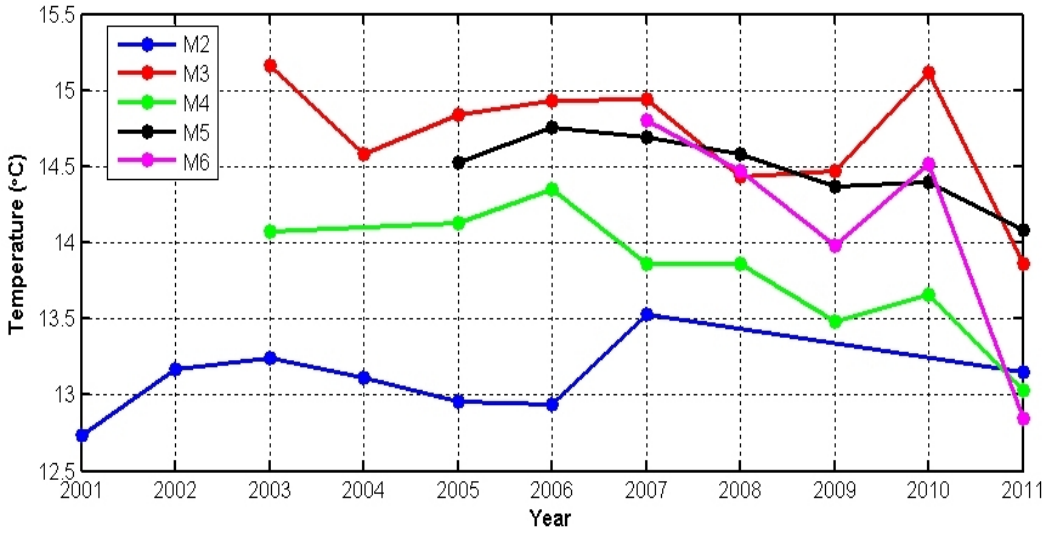

Figure 4.8 – M1-M5 Databuoys deployed around the Irish coast show that water temperatures were significantly lower in 2011 than in previous years

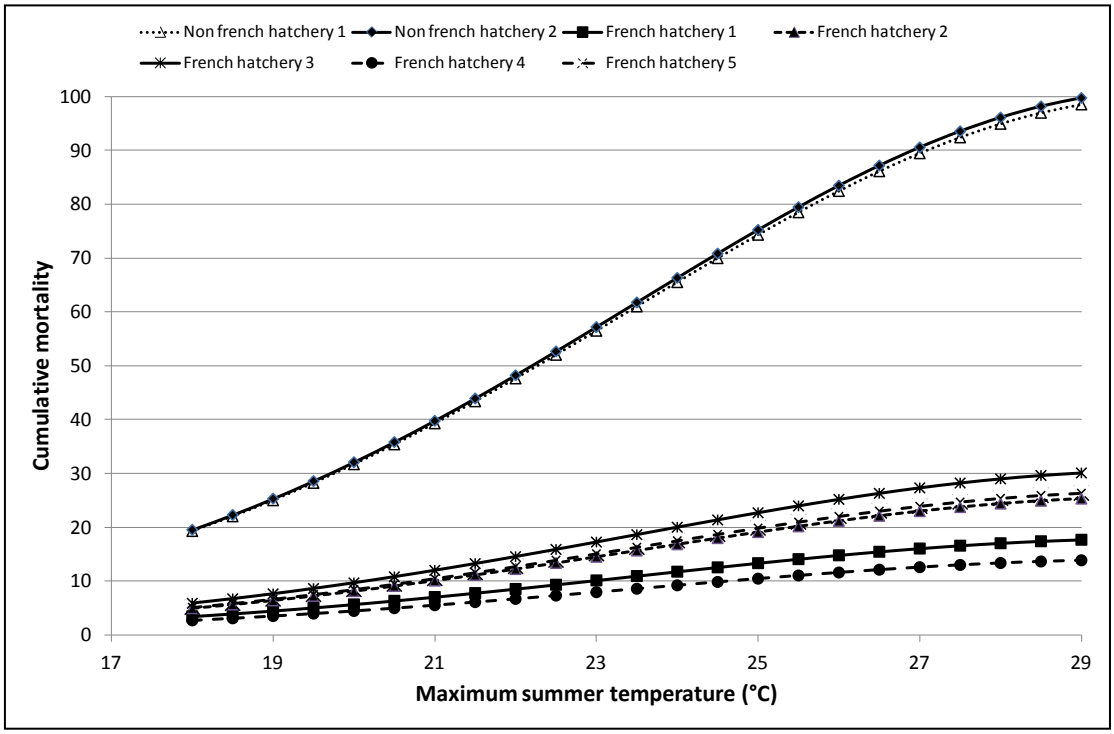

Figure 4.9. Predicted best linear unbiased estimator of the cumulative mortality from the multivariable mixed linear model plotted against the maximum summer temperature, for each hatchery in OsHV-1 µvar positive bays

4.4 Discussion & Conclusions

It has been suggested that increased water temperature could lead to a re-activation of latent infection (Sauvage et al., 2009). Analysis of temperature data recorded throughout the course of the study found a quadratic relationship between the natural logarithm of the cumulative mortality and the maximum temperature observed between June and August 2011. Unbiased linear estimates of mortality against the 'maximum summer temperature' indicated that mortalities increased with temperature until a temperature peak was reached. The maximum temperature observed during this study may have occurred as a one day event rather than a sustained rise in temperature.

Furthermore, visual assessment of environmental data for seven high mortality sites at which mortality and temperature data were available, temperature had exceeded 16°C in the week prior to mortalities at five of the sites. At the other 2 sites, temperatures had either exceeded 16°C in the previous month (Sherkin) or were around 16°C in the previous week (Woodstown Strand) (Appendix B). There was very little difference in temperature between the low and high mortality sites prior to the start of mortalities (Table 4.1).

Numerous studies have highlighted the multifactorial nature of mortality events in *C. gigas* oysters. The European Food Safety Authority (EFSA, 2010) noted the absence of significant mortality outbreaks when the water temperature had been below 16°C. Several studies have highlighted the potential role of increased water temperature in oyster mortality events, with OsHV-1 often being detected when temperatures increase quickly but no longer being detected when temperatures were stable even if the temperature remained high (Garcia et al., 2011). Evidence of such increases in water temperature associated with mortality outbreaks were found in the results of this study. Visual assessment results of temperature data for Trawbreaga, Achill Sound and Dungloe in particular demonstrate this, showing an increase in temperature coinciding with the time period of when mortality in *C. gigas* was reported (Appendix B).

In addition, according to data collected from M1-M5 Databuoys deployed around the Irish coast, water temperatures were significantly lower in 2011 than in previous years (Figure 4.8). In future investigations environmental data should be compared for consecutive years, to assess the relationship with *C. gigas* mortality events. This would provide further evidence to consolidate whether environmental changes, particularly significant changes in water temperature, had any impact during the onset of mortality events.

Conclusions

In the current study, a range of risk factors were considered relating to the local environment. This work was guided by international literature and also the findings (and recommendations) from an earlier Irish study Peeler et al., (2009). The primary conclusions of this investigation are as follows:

- There was very little difference in temperature between the low and high mortality sites prior to the start of mortalities (Table 4.1) and water temperature was not significant in the final multivariable model.
- Unbiased linear estimates of mortality against the 'maximum summer temperature' indicated that mortalities increased with temperature until a temperature peak was reached.
- The maximum temperature observed during this study may have occurred as a one day event rather than a sustained rise in temperature.
- According to data collected from M1-M5 Databuoys deployed around the Irish coast, water temperatures were significantly lower in 2011 than in previous years (Figure 4.8)

Chapter 5 – Discussion, Conclusions & Recommendations

5.1 OsHV-1 µvar Prevalence in Ireland

In this work, OsHV-1 µvar was found to be the most prevalent strain of Ostreid herpes virus associated with mortality outbreaks in *C. gigas* oysters in Irish bays. In Chapter 2, the prevalence of OsHV-1 µvar in Ireland was discussed.

The OsHV-1 µvar strain was shown by this study to be the predominant strain present in Ireland being detected in 29 OsHV-1 µvar infected bays. Although OsHV-1 µvar is the predominant genotype present and is detected in all 29 bays, co-infection with the genotype OsHV-1 µvar (with guanidine insertion) was observed in 6 of these 29 bays. A retrospective study was conducted on archive FFPE *C. gigas* material associated with mortality outbreaks prior to 2008 and found no evidence of the OsHV-1 reference strain. Molecular analysis using locked nucleic acid (LNA) real-time PCR method specific for the detection of OsHV-1 µvar found one FFPE sample to be OsHV-1 µvar positive from a cohort of *C. gigas* oysters collected in 2007.

Since OsHV-1 µvar was first detected in 3 bays in Ireland during 2008, efforts have been made to understand the spread of OsHV-1 µvar (Lynch et al., 2012; EFSA, 2010; Peeler et al., 2012, Peeler at al., 2009). Further work is needed in order to establish possible causes for the spread of OsHV-1 µvar. There is also need for further studies of the occurrence and potential transmission of OsHV-1 µvar virus, particularly in the area of vector species being potential source of OsHV-1 µvar infection.

The prevalence of OsHV-1 µvar in 29 bays around the coast is predominantly associated with mortality events in *C. gigas*. The cause of mortality events in *C. gigas* spat and larvae is multifactorial, with OsHV-1 infection (with OsHV-1 µvar now predominating) being a necessary, but not sufficient, cause (Burge et al., 2005; Garcia et al., 2011; Peeler et al., 2012; Samain and McCombie, 2008). An epidemiology study

conducted in Ireland during 2009 by Peeler et al., (2012) gave some insight into how management practices and a range of risk factors could be manipulated in order to reduce the risk of infection with OsHV-1 µvar and also made suggestions as to what should be deemed important for future studies. These suggestions were addressed in the second part of this study, which aimed to investigate risk factors associated with OsHV-1 µvar related mortality outbreaks in *C. gigas* in Ireland.

5.2 Mortality events in 2011 & Management Factors

Increased farmed oyster mortality has been an ongoing concern in Ireland for some years (Malham et al., 2009; Peeler et al., 2012), although there is evidence from the current study of a lower median batch mortality in 2011 (16%) compared with previous years; 2009 (37%) or 2010 (32%). The epidemiology study, described in Chapters 3 & 4, sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. From the analysis of these risk factors in 2011, several factors were found significant during mortality outbreaks, including hatchery source, and OsHV-1 viral load in 2011 and the maximum water temperature recorded between June and August 2011.

There was a strong association between hatchery source and mortality, with French hatchery seed having significantly lower mortality when compared with non-French hatchery seed. There were also differences evident between hatchery stocks, in relation to OsHV-1 µvar exposure. As the OsHV-1 µvar virus is now endemic in France (Pernet et al., 2012) French sourced stock would have been pre-exposed to the virus. As opposed to seed sourced from non-French hatcheries, which are located in areas outside of Ireland and have not been previously exposed to OsHV-1 µvar. This non-French stock would be naive to OsHV-1 µvar, and introduction of naive stock to endemically infected bays appear to have higher mortality rate compared to pre-exposed stock. It has been shown that prior exposure is likely to lead to a degree of latency (Degremont et al., 2013; European Food Safety Authority, 2010), a common feature of other herpesvirus infections (Arzul et al., 2002)

OsHV-1 µvar viral load was assessed during 2011 using a sampling strategy of 30 *C. gigas* oysters per bay; which provided 95% confidence that viral infection would be detected at a prevalence of 10% or greater if the virus were present. The viral load of OsHV-1 µvar in each participating bay was assessed during the study and OsHV-1 µvar status was found to be significantly associated with mortality. This finding is consistent with previous work, where it has been shown that there is an increased mortality risk with increasing quantities of OsHV-1 µvar DNA (Garcia et al., 2011; Pepin et al., 2008; Sauvage et al., 2009; Schikorski et al., 2011).

In the current study, a range of risk factors were considered, as outlined in Table 3.1, relating to farm management. This work was guided by international literature and also the findings (and recommendations) from the earlier Irish epidemiology study (Peeler et al., 2012). A range of other management factors were analysed as part of the study such as; Time in water, Bag mesh size, Age placed in water , Journey duration, Handling at grading, Position on shore, Bay, Sea bed type, Bag split, Bag turning, Average stocking density, Transport. All of these farm management risk factors were analysed but were found to be not significant in the current multivariable modelling, despite the role of farm management during mortality events having been emphasized by previous studies (Samain and McCombie, 2008; European Food Safety Authority, 2010). However, the final model only accounted for 24% of the variation in mortality, and therefore there are other risk factors that are not accounted for by the model.

5.3 Mortality events in 2011 & Environmental Factors

In addition to OsHV-1 µvar infection and management risk factors, a range of environmental risk factors have been implicated. As stated in Chapter 4, analysis of temperature data recorded throughout the course of the study found a quadratic relationship between the natural logarithm of the cumulative mortality and the maximum temperature observed between June and August 2011.

Furthermore, visual assessment of environmental data for seven high mortality sites at which mortality and temperature data were available, temperature had exceeded 16°C in the week prior to mortalities at five of the sites. At the other 2 sites, temperatures had either exceeded 16°C in the previous month (Sherkin) or were around 16°C in the previous week (Woodstown Strand) (Appendix B). There was very little difference in temperature between the low and high mortality sites prior to the start of mortalities (Table 4.1).

In addition, according to data collected from M1-M5 Databuoys deployed around the Irish coast, water temperatures were significantly lower in 2011 than in previous years (Figure 4.8).

5.4 Study limitations

There are a number of limitations to the current study which should be noted for future studies. Data collection was reliant on farmer recall to determine the start date of mortalities. This proved problematic, however, both in terms of data validity and completeness. The precision of these estimates is influenced by the frequency with which farmers check their stock. In most cases, due to accessibility on spring tides, stock can only be checked at best every 2 weeks, and in some cases stock may not be checked for up to a month. Similar challenges have been described in a similar study conducted among French oyster farmers (Lupo et al., 2012), where increases in reporting sensitivity were found to occur concurrent with outbreak occurrences and with the implementation of financial incentives to encourage farmer reporting of mortality events.

The final mortality data for each batch in the study was based on the outcome of grading for each batch in early spring 2012. Some farmers who had several batches included in the study, found it difficult to obtain grading data for individual batches as grading would usually be carried out on a stock basis rather than a batch basis. We also faced some gaps in the environmental data, noting that recordings were not taken during the same time period across all sites. In addition, although data were available on a range of environmental parameters, only the temperature data proved suitable for

subsequent analysis. Extra environmental data were only recorded at a limited number of sites due to cost and availability of monitoring equipment. There were some issues with tracking batches once they had been split. Several batches were split during the season, resulting in new stocking densities with new bag mesh sizes, and several new locations for the split batch. This proved problematic when tracking split batches through the season, as once the original batch was split; all subsequent batches had a separate set of data, and were essentially a new batch. Finally, the study batches were not chosen at random, primarily for logistical reasons. It is possible that these samples are not representative of the broader oyster farming population in Ireland.

5.5 Conclusions

- In this work, the OsHV-1 µVar strain was shown to be the predominant strain of Ostreid herpes virus associated with mortality outbreaks in *C. gigas* oysters, being detected in 29 OsHV-1 µvar infected Irish bays, along with another similar genotype of OsHv-1 µVar, with a guanidine insertion and substitution of a guanine, which was detected in 6 of the 29 OsHV-1 µvar infected bays.
- Only one OsHV-1 reference strain sequence was identified in one oyster producing bay in Ireland in 2009.
- Retrospective analysis of FFPE *C. gigas* material, did not find any evidence of OsHV-1 reference strain in samples collected before the OsHV-1 µvar outbreak in 2008. Further, one sample, collected in 2007, was found to be positive for the presence of OsHV-1 µvar.
- The completed epidemiology study provides some insights into mortality events affecting farmed Pacific oyster production in Ireland. The epidemiological study conducted found that batch mortality was lower in 2011, compared with earlier years, in association with lower OsHV-1 µvar viral loads.
- It was also evident that mortality was significantly associated with hatchery source and ploidy, for reasons that are currently unclear.
- Future studies should aim to investigate the prevalence of OsHV-1 µvar sourced from various overseas hatcheries that would be re-laid in Irish bays under similar

husbandry and environmental conditions.

- Temperature data analysis indicated that mortalities in *C. gigas* oysters increased with temperature until a temperature peak was reached. The maximum temperature observed during this study may have occurred as a one day event rather than a sustained rise in temperature. Throughout the study, water temperatures were significantly lower in 2011 than in previous years.
- As there was 24% variation in mortality in the final model suggests there are other risk factors not accounted for by the model. Future studies could include other risk factors that were not included in the study, such as investigating the role played by *Vibrio* species during mortality outbreaks.

5.6 Recommendations & Suggestions for Future Research

Further Epidemiology Studies

Further work is needed to elucidate the basis for these findings from this current epidemiology study, including the relative importance of prior exposure to OsHV-1 µvar and the effect of oyster ploidy.

The objective of an investigation, which took place in 2012, was to concentrate on tracking *C. gigas* oyster seed that was sourced from various overseas hatcheries and re-laid in 7 OsHV-1 µvar infected bays (Carlingford, Donegal, Dungarvan, Clew Bay, Gweedore, Sherkin, and Castlemaine) under similar husbandry and environmental conditions during 2012.

The study concentrated on tracking *C. gigas* oysters on a stock basis throughout the season, rather than a batch basis, as this approach allowed for an overall assessment of the whole stock from each hatchery source as opposed to one batch from each hatchery source thereby allowing the gathering of composite data for all the batches from that one source, rather than focusing on the survival of individual batches.

A longitudinal sampling protocol to examine the prevalence of OsHV-1 µvar was also carried out in conjunction with the environmental monitoring on all sites.

The epidemiological analysis of data collated during the 2012 study will be carried out by CVERA, UCD.

Investigate other risk factors

As there was 24% variation in mortality in the final model, this suggests there are other risk factors not accounted for by the model. Future studies should include other risk factors that were not included in the study, such as investigating the role played by *Vibrio* species during mortality outbreaks. *Vibrio* related species, *Vibrio splendidus*, *Vibrio aestuarianus* and *Vibrio harveyi* have frequently been observed in association with mortality outbreaks prior to and after the 2008 OsHV-1 µvar outbreak.

Several recent mortality outbreaks affecting adult and half-grown *C. gigas* oysters in France and Ireland have been associated with the detection of *Vibrio* species i.e. *V. splendidus* and *V. aestuarianus* in *Crassostrea gigas* adult oysters, which would suggest that further research is required in this area as it appears to be an emerging significant pathogen.

Transmission Pathways

Previous work has established the presence of OsHV-1 µvar in mussels in Ireland, which has provided evidence for the need for further studies of the occurrence and potential transmission mechanisms of the OsHV-1 µvar virus.

The potential role of depuration centres in the introduction of OsHV-1 µvar has also been highlighted as a transmission mechanism of infection. In 2012, an outbreak of OsHV-1 µvar in a previously un-infected bay in Ireland was linked to mussels which had been moved from an OsHV-1 µvar infected area to a nearby depuration centre. This highlights the need for further investigation into the occurrence and potential transmission mechanisms of the OsHV-1 µvar virus.

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Appendix A: Data Collection Sheets

Investigation of Summer Mortality *in oysters (*Crassostrea gigas*) in Ireland 2011*

Notes for Officer Compiling data

1. Contact details for any queries when filling in form <*not included here*>
2. Sections 1 & 2 should be completed on the first visit with the farmer. Ask the farmer to have the hatchery invoices as well as the health cert to hand in advance of going out. The former provides the best source of information on the seed.
3. If possible, when the selected batches are split try to visit the farm to ensure maximum traceability. Details of new stocking densities at the time of the split should be recorder in Section 4.2 (and 4.3 if seed was actually graded).
4. Section 3 and Section 4 should be completed as soon after the mortality event as possible.
5. Ask the farmer to keep a sample of the dead seed for you to measure at a later date. This is to see if there is any correlation between size and mortality. Ideally the farmer should keep a sample from each batch labelled for you.
6. Section 5 will be completed when the farmer grades his seed.

Other points to remember:

7. One form should be completed per oyster batch / sub-batch
8. A sub-batch is created prior to mortality when/if the batch is split, one or more proportions of the original stock is moved to a location where its exposure to the virus might be altered in some way e.g. it may be moved higher or lower on the shore or there may be batches of commercial stock separating it from the original batch.
9. To identify a sub-batch: French hatchery 1/16.05.11/A or JP2M/04.06.11/B
10. Where a participating farmer has more than one batch and is known to be good at record keeping and "batch control", try to follow more than 1 batch. When deciding which batches to follow, consider parameters such as diploid/ triploid, wild/ hatchery, different hatcheries, early seed/ late seed (potentially even seed which came in last year subsequent to the 2010 outbreak), different locations on the strand etc. The idea is to capture as much "difference" as possible, to see if we can measure the impact.

Keep regular contact with all participating farmers. Don't expect them to remember to call you!

Investigation of *Summer Mortality* in oysters (*Crassostrea gigas*) in Ireland 2011

Oyster batch data collection form – **one form per oyster batch/sub-batch to be completed**

1.0 Batch Identification Data

Name of Farm:

_____

*Name of Operator:*_____

*Name of site and/ or name of Bay:*_____

Batch number _____ *(generated by project manager)*

*Batch Identifier: (e.g French hatchery 1, 16.05.11)*_____

*Quantity of oysters in the batch/ sub-batch to be followed:*_____

*Number of bags the batch/ sub-batch has been placed in:*_____

Has original batch been divided and moved to different locations? (Y/N): _____

If so, how many sub-batches were created? _____

*Batch Location (4GPS extremities): 1*_____ *2*_____ *3*_____ *4*_____

Position on shore (high / mid / lower): _____

2.0 Factors of interest.

Batch Level:

- Date placed in water (dd/mm/yy): _____
- Method of transport used from site of origin to this site: _____
- What was the duration of the journey from hatchery? (hours) _____
- Average size & weight placed in water (e.g. T/G number plus weight in grammes): _____
- Stocking density when placed in water (seed/bag): _____
- Bag mesh size (mm): _____
- Ploidy (diploid vs. triploid): _____
- Hatchery vs. wild caught (H or W):_____
- Hatchery Name: _____
- Bay / region where seed originated (if wild): _____
- Handling at grading (mechanical or manual or both?): _____
- Geology/seabed type (e.g. hard sand, soft sand, gravel, mud etc):_____

-

3.0 Preliminary Oyster Batch Mortality Data

(NOTE: This data should be collected as soon after mortality event as possible – the information is based purely on the opinion of the farmer, rather than on the verification of data by us):

Date data collected: _____

When did mortalities start on site? (date): _____

What is your estimate of the % mortality overall in your:

Seed: _____

Half Grown: _____

Adults: _____

Did mortality occur in the batch/sub-batch of interest? _____

If so, when did the mortality start in that batch: _____

What is your estimate of the % mortality in the batch/sub-batch of interest?

_____

Average size of seed at the onset of mortality (e.g. T/G number plus weight in grammes): _____

Note: ask the farmer to keep a sample from each batch for later verification by BIM/MI – See Annex 1

When did mortalities finish on site? (date): _____

When did mortalities finish in batch of interest? (date): _____

In the farmers opinion were there any predisposing events to the mortalities? (e.g. storms, silting, algal blooms, predators, heavy rainfall, anoxia, water discolouration, other?)

_____

_____

_____

Was there any movement of equipment (including bags) from other farms/hatchery onto site, this year?

_____

_____

_____

Was the mortality in 2 or more waves? (Y/N) ____ How many waves? _____

Was the mortality experience the same across the site? (Y/N) _____

If not, provide details:_____

_____

Provide details of "normal" background mortality levels for this site (i.e. before OsHV-1 µvar):

Seed: _____

Half grown: _____

Adults: _____

Any other comments that the farmer thinks is relevant to the disease presentation observed on his/ her site, this year:_____

4.0 *Handling prior to and/or during mortality*

4.1 What was frequency of bag turning per month (i.e. turns per month, in the batch/ sub-batch of interest):

April _____
May _____
June _____
July _____
August _____
September _____
October _____

Did turning of oysters continue during mortality episode? (Y/N): _____

4.2 Table 1: Please complete splitting data for the batch/ sub-batch of interest.

Date of splitting	No of bags to be split	Original stocking density	New bag mesh size	New stocking density	No of bags returned to the shore	Time out of the water

GPS CO-ORDINATES OF NEW (EXPANDED) BATCH LOCATION:
Extremity 1: _____ Extremity 2: _____
Extremity 3: _____ Extremity 4: _____

4.3 Table 2: Please complete grading data for the batch/ sub-batch of interest if grading has taken place in advance of the occurrence of OsHV-1 µvar related mortalities in summer 2011:

Date of Grading: _____

			Large Grade			Middle Grade			Small Grade		
# Bags Brought In	S.D	Time Out of Water (hours)	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.

Date of Grading: _____

			Large Grade			Middle Grade			Small Grade		
# Bags Brought In	S.D	Time Out of Water (hours)	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.

GPS CO-ORDINATES OF NEW (EXPANDED) BATCH LOCATION:
Extremity 1: _____ Extremity 2: _____
Extremity 3: _____ Extremity 4: _____

Note: Any grading prior to mortality will result in sub-batches because stocking density and size of oysters may play a role in any subsequent mortality

5.0 <u>Final Oyster Batch (or sub-batch) Mortality Data</u>

To be collected at the first grading which occurs after OsHV-1 µvar related mortalities in 2011 (may be in autumn 2011 or early in 2012):

Date of grading: _____

# bags brought in	Bags Returned Large Grade			Bags Returned Middle Grade			Bags Returned Small Grade		
	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.
Quantity of live seed returned to shore per grade (adjusted for shell content in small and medium grade – See Annex 2)									

Date of grading: _____

# bags brought in	Bags Returned Large Grade			Bags Returned Middle Grade			Bags Returned Small Grade		
	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.	# Bags	Mesh size (mm)	S.D.
Quantity of live seed returned to shore per grade (adjusted for shell content in small and medium grade – See Annex 2)									

Overall quantity of seed in (sub)batch returned to shore	
Initial quantity of seed in (sub)batch deployed	
% Mortality in (sub)Batch	

ANNEX 1

DATE	NO OF OYSTERS MEASURED	AVERAGE LENGTH AT COMMENCEMENT OF MORTALITY

ANNEX 2

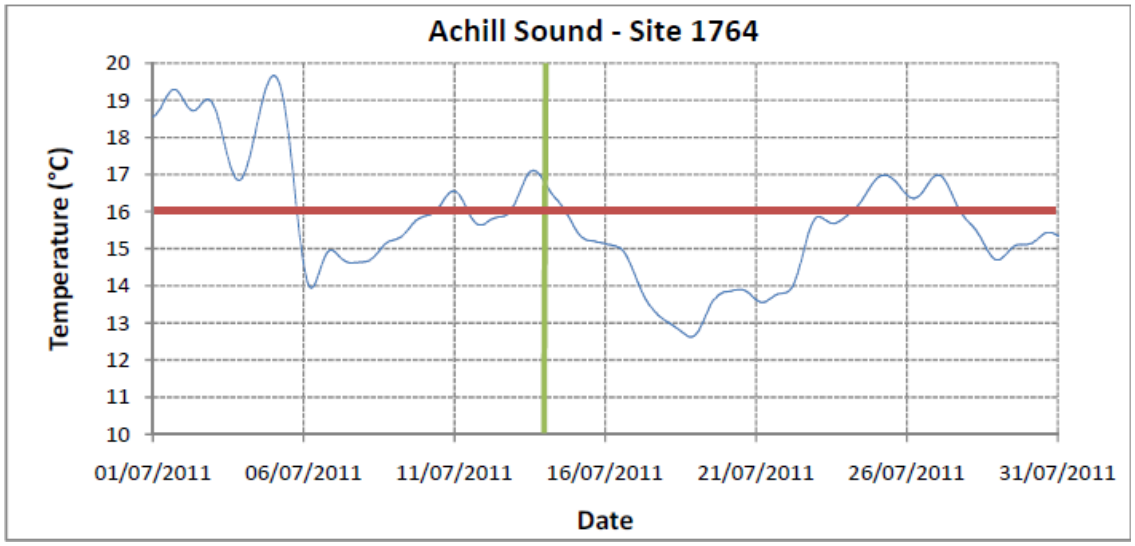
Method for estimating quantity of live seed in middle and small grade where a lot of dead shell is returned to the bag:

- After seed has been re-bagged, select 3 bags
- While immersing the bag in water, gently shake the contents to ensure as representative a sample as possible and take a handful of seed from the bag.
- Count the number of live shells in the sample, ensuring a minimum of 50 (if you don't get 50 live take another handful using the same method)
- Weigh the entire sample and weigh the overall contents of the bag.
- Total number of live animals = (Total weight / sample weight) X number live in sample
- This should be repeated in the 3 bags and an average count taken

Appendix B: Visual Assessment of Environmental data

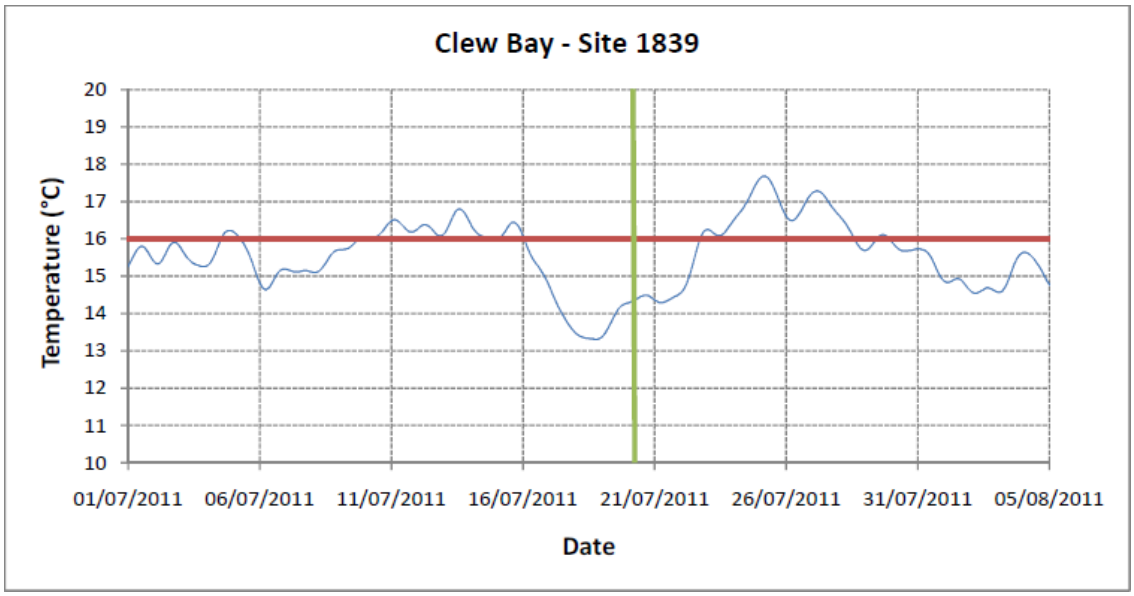
Visual Assessment Results

For seven sites where significant high mortalities were reported in 2011 (sites Dungloe, Woodstown, Achill Sound, Lough Swilly, Sherkin, Trawbreaga and Clew Bay), a visual assessment was made of temperature changes during the period when mortality events were first recorded. The final high mortality site (Ballymacoda Bay, site 1772) was excluded from this assessment as there was no temperature data recorded during the time period around the reported start of mortality.



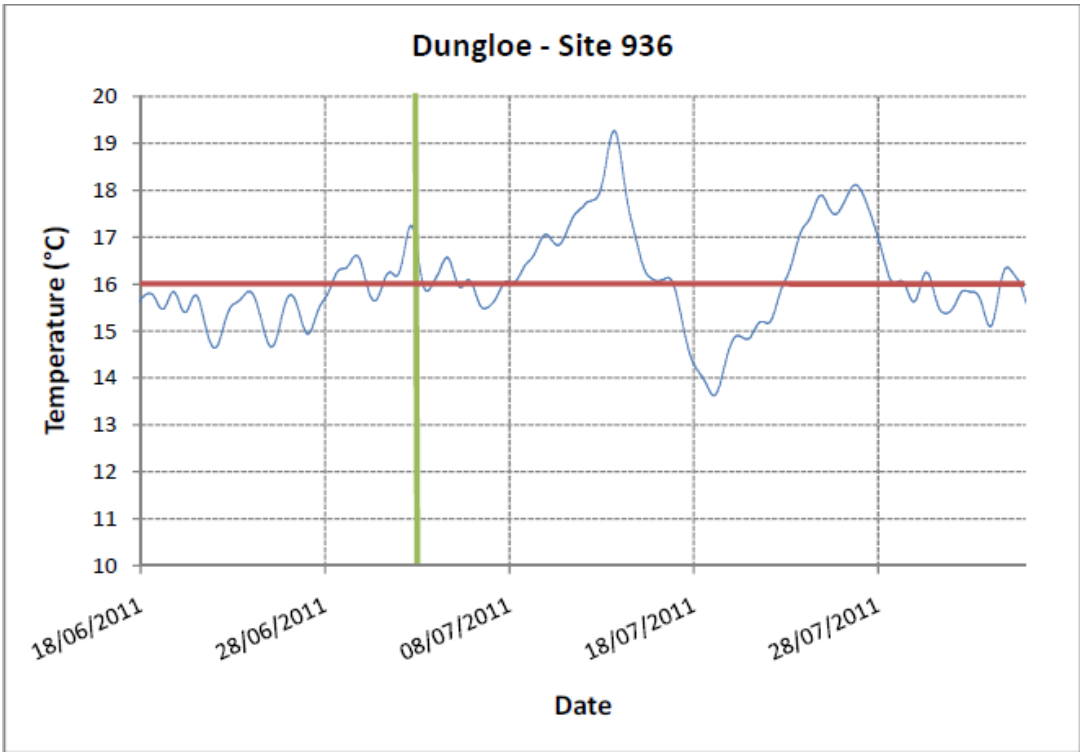
a. Achill Sound (Site: 1764)

The onset of mortality was recorded as 14 July 2011 for 3 batches of oysters on the same site in Achill. All 3 batches were from 3 different hatchery sources (non-French hatchery 1 (diploid), French hatchery 1 (triploid), non-French hatchery 2 (diploid)), and were all seed imports of 2011. All batches of seed experiencing mortality were of similar size of 10-25mm. The 'non-French' batches experienced similar levels of mortality (Non-French hatcheries 1 and 2: 74% and 76%, respectively). The French batch had considerably less mortality with only baseline mortality levels of mortality being reported for seed from French hatchery 1 at 13%. Mortality was recorded to have ceased by 31 July 2011, indicating that the mortality event lasted 15 days. There was a recorded increase in temperature, to 17°C, the day prior to mortality being reported.



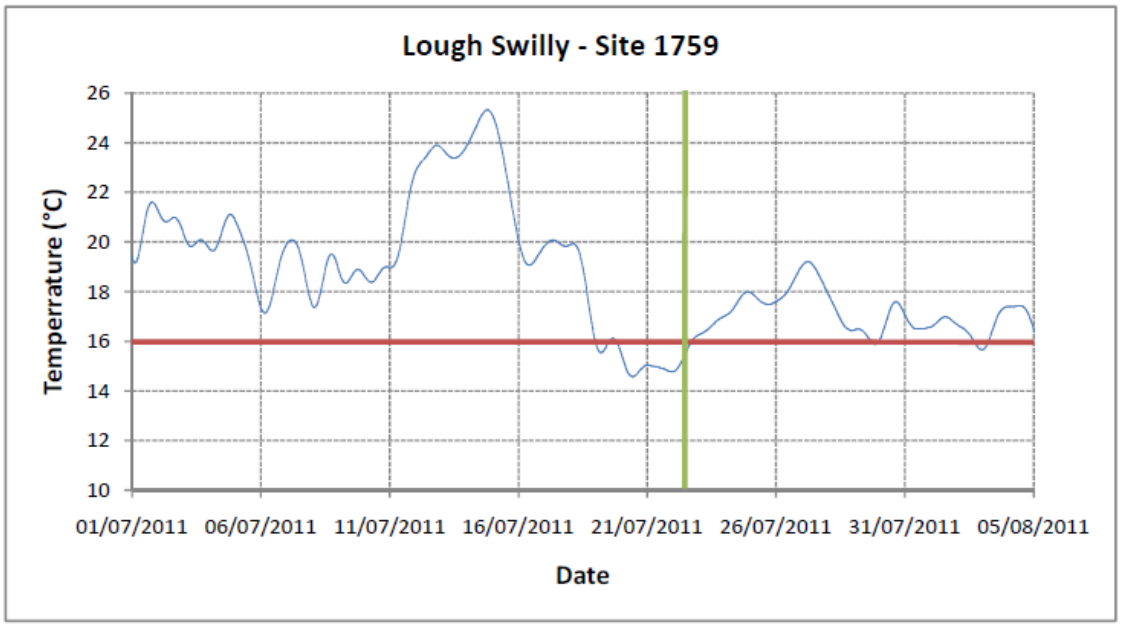
b. Clew Bay (Site: 1839)

The onset of mortality was recorded on 20 July 2011 for 1 batch on one site in Clew Bay. The percentage mortality was 35%. A further batch was subsequently moved to another site in Clew Bay, and was reported to have 57% mortality after being moved. The batch at this site was from French hatchery 5, imported in early 2011. The batch of seed was 10mm in size and triploid. There was a sharp drop in temperature prior to mortality being reported, followed by a gradual increase in temperature where the 16°C threshold was exceeded, the week during which the onset of mortality was first noticed.



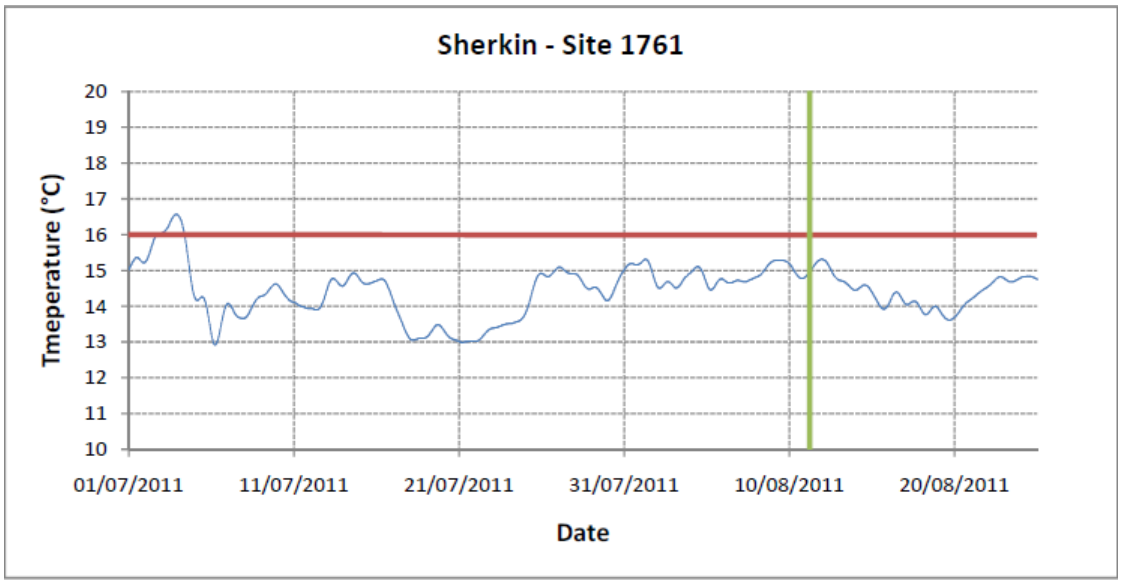
c. Dungloe (Site: 936)

The onset of mortality was recorded as 'the first week in July', so start date for mortality was taken as 3 July 2011. Mortality was reported for 3 batches on 2 different sites. The percentage mortality ranged from 15-90%. All seed in the batches concerned were French hatchery seed from 2011, the batch with the highest mortality (90%) was diploid seeds and the other 2 batches were triploid, all batches were 6mm when the mortalities began. A substantial increase in temperature was observed approximately one week after mortality is reported to have started. The onset of mortality coincided with the first instance of temperature exceeding 16°C. The mortality event was reported to have lasted for 2 weeks.



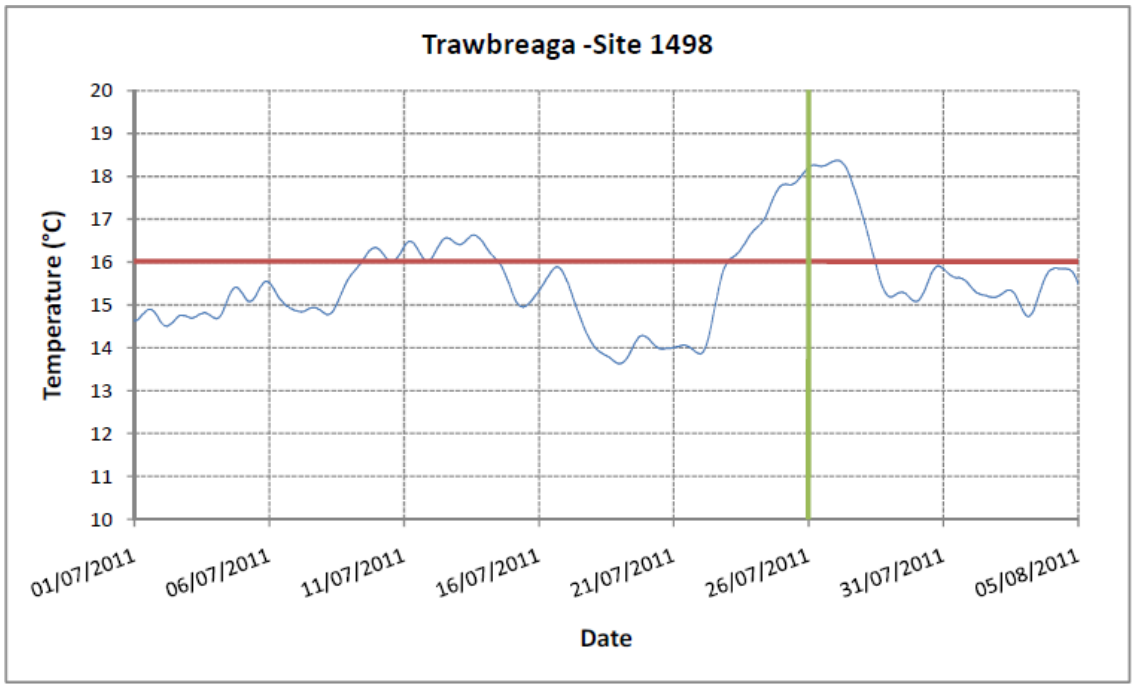
d. Lough Swilly (Site: 1759)

The onset of mortality was recorded on the 22 July 2011 for 3 batches on the same site in Lough Swilly. All 3 batches were French sourced seed, which had been imported in early summer of 2011. The overall percentage mortality ranged between 2-38% for the 3 batches concerned. The temperature trend for this time period shows that there had been considerably elevated temperatures, where the temperature exceeded 16°C, for a number of weeks prior to the mortality outbreak.



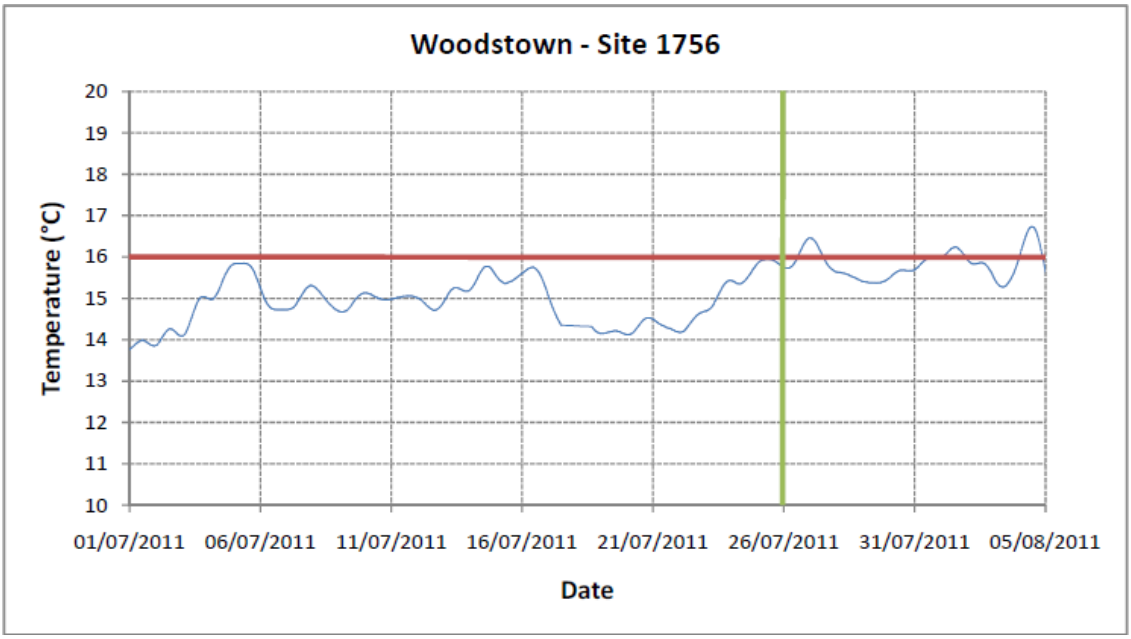
e. Sherkin (Site: 1761)

The onset of mortality was recorded on 11 August 2011. There was only one batch in question that reported mortality, this batch was diploid seed sourced from non-French hatchery 1, was 6 mm at the start of mortalities and had 86% mortality. There was no clear association between the start date for the onset of mortality and any substantial change in the temperature data at that time.



f. Trawbreaga Bay (Site: 1498)

The onset of mortality was recorded on 26 July 2011 for 2 batches on the same site in Trawbreaga. The percentage mortality reported for both batches concerned was 99% and 100%, respectively. Both batches were sourced from non-French hatchery 1, with one batch being triploid and the other batch being diploid and were 4mm in size at the start of mortalities. The mortality was reported to have ceased on 10/8/11, suggestive of a mortality event lasting about 3 weeks. The temperature data recorded when mortality was reported to have started shows a clear peak at 18°C.



g. Woodstown Strand (Site: 1756)

The onset of mortality was recorded on 26 July 2011 for 2 batches of oysters on the same site in Woodstown. The overall percentage mortality ranged from 32-48%. All batches that were reported as experiencing mortality were ~6mm in size, and were triploid seed that was imported from the same French hatchery (French hatchery 1) in the same week in August 2010. The temperature data recorded shows that there was a gradual increase in temperature in the week preceding the mortality outbreak. The temperature exceeded 16°C during the week that the mortality outbreak was reported.

Appendix C: Published work

Risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. Prev. Vet. Med. 113, 257–267.

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Risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011

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ABSTRACT

The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland. Episodes of increased mortality in *C. gigas* have been described in many countries, and in Ireland since 2008. The cause of mortality events in *C. gigas* spat and larvae is suspected to be multifactorial, with ostreid herpesvirus 1 (OsHV-1, in particular OsHV-1 µvar) considered a necessary, but not sufficient, cause. The objectives of the current study were to describe mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and oyster endogenous factors. A prospective cohort study was conducted during 2010–2012, involving 80 study batches, located at 24 sites within 17 bays. All 17 bays had previously tested positive for OsHV-1 µvar. All study farmers were initially surveyed to gather relevant data on each study batch, which was then tracked from placement in the bay to first grading. The outcome of interest was cumulative batch-level mortality (%). Environmental data at high and low mortality sites were compared, and a risk factor analysis, using a multiple linear regression mixed effects model, was conducted. Cumulative batch mortality ranged from 2% to 100% (median = 16%, interquartile range: 10–34%). The final multivariable risk factor model indicated that batches imported from French hatcheries had significantly lower mortalities than non-French hatcheries; sites which tested negative for OsHV-1 µvar during the study had significantly lower mortalities than sites which tested positive and mortalities increased with temperature until a peak was reached. There were several differences between the seed stocks from French and non-French hatcheries, including prior OsHV-1 µvar exposure and ploidy. A range of risk factors relating to farm management were also considered, but were not found significant. The relative importance of prior OsHV-1 µvar infection and ploidy will become clearer with ongoing selection towards OsHV-1 µvar resistant oysters. Work is currently underway in Ireland to investigate these factors further, by tracking seed from various hatchery sources which were put to sea in 2012 under similar husbandry and environmental conditions.

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1. Introduction

The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland, both in terms of volume and value, with an annual production of over 7000 metric tonnes (Bord Iascaigh Mhara (BIM)/the Irish

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Sea Fisheries Board, personal communication). With a current value of €28–30 million per year, *C. gigas* accounts for approximately 20% by volume of overall shellfish produced in Ireland (BIM, personal communication). The main method of cultivation for Pacific oysters used in Ireland is bag and trestle cultivation, which is an off-bottom culture method. This cultivation method allows the oysters to be placed in mesh bags on metal framed structures called trestles in the inter-tidal zone, which allows access to the stock during low tide (Tidwell, 2012). Over half of all current licensed aquaculture producers in Ireland are oyster farmers, with *C. gigas* being grown in 44 bays all around the coast. *C. gigas* seed is predominantly sourced from hatcheries/nurseries or harvested wild seed, which is imported mainly from France and to a much smaller extent from the UK and the Channel Islands. The main export market for Pacific oysters is France, although there is also a growing market in Asia.

Since the 1950s, episodes of increased mortality in *C. gigas* have been described globally in all areas of production. In Europe, severe mortality events in cultured Pacific oyster were observed during the summers of 2008 and 2009 (Dégremont et al., 2013). These events have been grouped by life stage into summer mortality in adults, mortality in spat and hatchery-related mortality (European Food Safety Authority, 2010). Mortality in spat and larvae at hatcheries have been associated with detection of ostreid herpesvirus 1 (OsHV-1), a virus also associated with mortality in other farmed bivalves, including the European flat oyster (*Ostrea edulis*), scallop (*Pecten maximus*) and the Manila clam (*Ruditapes philippinarum*) (Renault and Arzul, 2001; Arzul et al., 2002; Batista et al., 2007). In 2000, OsHV-1var, a variant strain of OsHV-1, was identified in French hatcheries (Arzul et al., 2001a,b). Although OsHV-1var presents several modifications in the C region of the genome, where the most significant modifications in relation to OsHV-1 occur, and a 2.8 kb deletion, OsHV-1 and OsHV-1var are considered representative of a single viral species as the differences between the two genotypes were not great enough to establish a separate viral species (Arzul et al., 2001b). In 2008, the emergence of a third strain was described, OsHV-1 µvar, in association with abnormal mortality in *C. gigas* in France (Segarra et al., 2010; Dégremont et al., 2013). It has since been shown that mortality in *C. gigas* spat can be induced following experimental infection with OsHV-1 µvar (Schikorski et al., 2011). Further, mortality can be induced following horizontal transmission of infection from unselected asymptomatic adult to juvenile *C. gigas* (Dégremont et al., 2013). Since 2009, OsHV-1 µvar has been the predominant herpes virus strain during mortality events (European Food Safety Authority, 2010). It is now believed that the cause of mortality events in *C. gigas* spat and larvae is multifactorial, with OsHV-1 infection (with OsHV-1 µvar now predominating) a necessary but not sufficient cause (Samain and McCombie, 2010). Other suspected risk factors include an increase or a sudden change in the temperature, husbandry practices such as introduction of possibly infected spat, and the movement and mixing of populations and age groups (European Food Safety Authority, 2010; Garcia et al., 2011). The European Food Safety Authority (2010) also noted that no outbreaks had been reported when the water temperature was below 16 °C.

In Ireland, mortality events in *C. gigas* have been reported for some years (Malham et al., 2009), but linked, since 2008, to the presence of OsHV-1 µvar (D. Cheslett, personal communication). In that year, reports of mortality in Pacific oysters were received from three oyster producing bays, and the presence of the OsHV-1 µvar was confirmed in all three bays by PCR and sequence analysis of the amplicon (European Food Safety Authority, 2010). In 2009, extensive mortality and the presence of OsHV-1 µvar were reported from 15 oyster production areas, peaking in July with an average batch mortality of 37% and with mortality occurring, on average, over an 18 day period (Peeler et al., 2012). Although few clear associations between mortality and management factors were identified, the age of oysters when first infected with OsHV-1 µvar, the condition of the oysters, temperature, and other environmental factors each appeared important (Peeler et al., 2012). European Union legislation was subsequently introduced to prevent the spread of the virus to unaffected areas in Ireland and the United Kingdom, whilst still allowing trade to continue between infected areas (European Community, 2010), but noting that there was no realistic prospect of eliminating the virus (Peeler et al., 2010). OsHV-1 µvar related mortality has continued in Ireland each summer since the initial detection of OsHV-1 µvar in 2008, both in bays previously infected with this virus and coincident with spread of infection to new bays.

The objectives of the current study were to describe any mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and endogenous oyster factors, thereby providing information which could assist oyster farmers in minimising batch mortality in endemically affected areas.

2. Materials and methods

2.1. Study design and population

A prospective cohort study was conducted during 2010–2012, from the time of first batch immersion (03 August 2010), when oysters were 2 mm in size. The oysters were followed until the date of last batch grading before data analysis, in spring/summer 2012 (12 April 2012), where oysters had been immersed for up to 18 months (subsequently termed the study period). The oyster batch was the unit of interest, and was defined as a variable number of oysters of similar size, originating from one hatchery, placed at a particular site within a bay at one point in time. All 405 batches of farmed oysters that were in the sea in Ireland during 2011 were considered for inclusion in the study. Farms were selected for logistical reasons, such as the accessibility of stock to allow for frequent monitoring and the capacity to monitor individual batches among the stock throughout the season. From the selected farms batches were chosen in order to include batches from different hatcheries, ploidy status and immersion date within each bay.

The number of batches required for the study was estimated, based on a confidence interval of 95% (alpha = 0.05),

a power of 80% and assuming a mortality of 43% amongst batches in OsHV-1 positive bays and 12% in OsHV-1 negative bays (based on reported site-level mortality from the 2009 Irish survey (Peeler et al., 2010); data as presented for OsHV-1 positive bays and as estimated for OsHV-1 negative bays). The initial sample size was calculated using the following formula to detect a difference between two proportions (Dohoo et al., 2009):

$$n = \frac{\left[Z_\alpha\sqrt{(2pq)} - Z_\beta\sqrt{((p_1q_2)+(p_2q_2))}\right]^2}{(p_1-p_2)^2}$$

where $Z_\alpha = 1.96$, the value required for a confidence of 95%, $Z_\beta = -0.84$ the value required for a power of 80%, p_1 = estimate of lower proportion of disease, p_2 = estimate of higher proportion of disease, $q_1 = 1 - p_1$, $q_2 = 1 - p_2$, $p = (p_1 + p_2)/2$ and $q = 1 - p$.

The sample size was further adjusted to account for other confounders. Assuming other confounders were not strong confounders the sample size was increased by 15% (Dohoo et al., 2009, p. 50). Clustering within farms was accounted for using the following formula:

$$n' = n(1 + \rho(m-1))$$

where ρ is the intra-cluster correlation coefficient, assumed to be 0.5 and m is the average number of batches of oysters per farm, assumed to be 3. In total, the aim of the study was to sample around 80 batches.

2.2. Data collection

An initial survey of all study farmers was conducted to gather relevant data on each study batch, and to provide a framework for data collection at batch-level throughout the study period. The survey was administered in a joint effort by BIM regional officers and the Fish Health Unit of the Marine Institute (MI). Each study batch was tracked throughout the study period. To achieve this, BIM Regional Officers made regular visits to survey the batches and collect data on batch identification, risk factors of interest, batch mortality data, splitting, grading and handling frequency. For any batch where mortality occurred, details of the mortality event were recorded (i.e. start and finish date of the mortality episode, estimated percentage mortality in the batch, any predisposing factors). Data were recorded on specifically designed survey forms which were submitted to the MI and entered into a Microsoft Access database. Data collection sheets are available in Appendix A.

Samples of the stock (30 animals from one batch in each bay; Table 1) were tested for the presence of OsHV-1 in the Fish Health Unit laboratory at the Marine Institute. Cell lysis and nucleic acid extraction was carried out using QIAamp DNA Mini Kit (Qiagen) using Qiagen QIAcube, according to the manufacturer's instructions. DNA extracts obtained were subjected to an initial screening process for the presence of OsHV-1 using real-time PCR analysis based on a Sybr Green chemistry with C9/C10 primer set targeting the C region of the genome (Pepin et al., 2008). Confirmatory testing for OsHV-1 µvar was carried out using nested PCR where subsamples of real-time positive PCR products were analysed using nested conventional PCR, using C2/C6 primers (Arzul et al., 2002) in the first round of conventional PCR, and internal primer set F-int/R-int, with a 514 bp expected product size in the second round of conventional PCR, using PCR conditions (unpublished, D. Stone, CEFAS, UK).

A total of 55 data loggers, at least one per bay, were deployed to obtain environmental data. These were deployed in May 2011 on a small number of sites, due to availability of equipment, with further deployments made in early June 2011 on the remaining sites. Five different types of data loggers (two large types and three small types) were deployed, each recording a number of different parameters. The smaller data loggers were deployed attached to the bags in the batch of interest, whereas the larger data loggers were deployed alongside the trestles which held the batch of interest. These data loggers were programmed to record environmental parameters every hour for the duration of the deployment, with regular maintenance and calibration on a monthly basis. An illustration of the location of data loggers at Dungarvan bay is shown in Fig. 1.

Table 1
Independent variables considered in the study.

Independent variable	Explanation
Time in water	Measured in days from date of immersion until date of final batch grading. Also grouped into time when placed in water *(2010, Spring 2011 and Summer 2011)*
Age placed in water	*2–3, 4–7, >7 (months)*
Ploidy	*Diploid, triploid*
Bay	17 bays
Position on shore	*Low, mid, high*
OsHV-1 µvar bay status[a]	OsHV-1 µvar detection in 2011 *(positive/negative)*
Bag turning	Number of times each bag turned between May to August 2011
Transport	*Air, sea and road, road*
Journey duration	*≤24 h, >24 and <36 h, ≥36 h and <42 h, ≥42 h*
Bag mesh size	*<4, 4 and >4 (mm)*
Hatchery	The 7 hatcheries from which the oysters originated
Handling at grading	*Manual, mechanical, both*
Sea bed type	*Gravel, gravel/sand, mud, mud/sand, sand*
Average stocking density	*Average no. of oysters per bag.* If bags were split, oyster density was as estimated at the end of May 2011
Bag split	Whether bags were split during the study into smaller number of oysters (*yes/no*)
Maximum summer temperature	Maximum temperature reached from the start of June until the end of August 2011, measured at 23 sites with between 1 and 8 batches at each site.

[a] Sampling was carried out on a bay basis (i.e. 30 animals from one batch in each bay was sampled for the presence of OsHV-1, the sample size was based on detecting a design prevalence of 10% or greater with 95% confidence), however, the batch sampled may not have been part of this study. A bay was considered positive if at least one batch was infected.

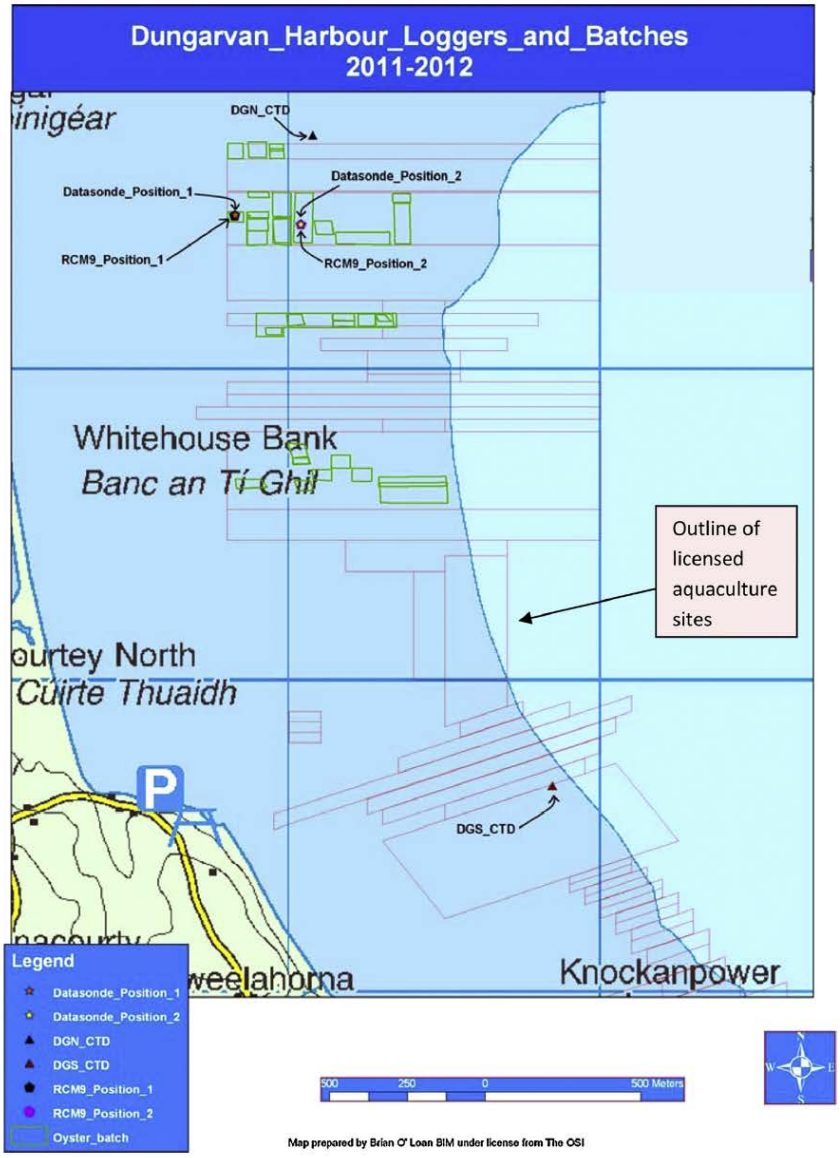

Fig. 1. The location of study batches and environmental data loggers (Hydrolab DS5 Multiparameter Datasonde loggers; _CTD loggers (DGS – Dungarvan South and DGN – Dungarvan North); RCM9 loggers) in Dungarvan Bay during 2011–12.

2.3. Descriptive analysis of temperature at high mortality sites where start date of mortality recorded

A high mortality site was defined as a site where 1 or more batches experienced a cumulative batch-level mortality > 34% (being the upper mortality quartile) during the study period. A low mortality site was defined as a site where no batches experienced a cumulative batch mortality > 34%. At all high mortality sites with available data (reported start date of mortality event and temperature data during the 30 preceding days), the median temperature within the previous 7 and 30 days prior to the recorded start of mortality were reported. At all low mortality sites, the median temperature during the same time periods as

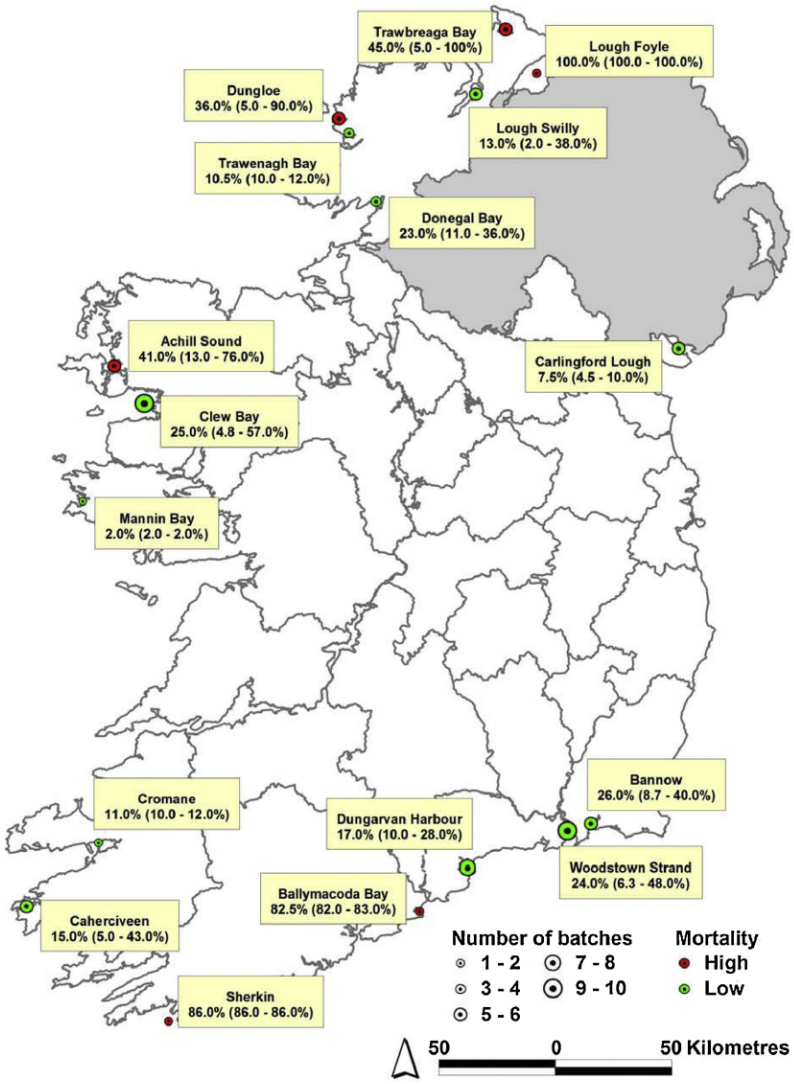

Fig. 2. Location of the 80 study batches and 17 bays, and the cumulative batch mortality (average, range) in each bay, of farmed *C. gigas* in Ireland during summer 2011.

Table 2
The median and interquartile range of temperature recorded within 7 and 30 days of the reported start of mortalities at seven high mortality sites (those with 1 or more batches with a cumulative mortality > 34%) during summer 2011 in Ireland. The median and interquartile range of temperatures at the ten low mortality sites (those sites without any high mortality batches) during the same time periods is included for comparison.

Bay[a]	Reported start of mortality	At each high mortality site Temperature			Among the ten low mortality sites Temperature		
		Median	Interquartile range		Median	Interquartile range	
			Q1	Q3		Q1	Q3
Within 7 days of start of mortalities							
Achill Sound	14-Jul-11	15.9	14.9	16.7	15.3	14.5	16.5
Clew Bay	20-Jul-11	14.8	13.9	15.8	15.0	13.7	16.7
Dungloe	03-Jul-11	15.7	15.3	16.9	14.4	13.5	15.7
Lough Swilly	22-Jul-11	18.8	15.5	20.0	14.4	13.4	15.4
Sherkin	11-Aug-11	14.8	14.5	15.1	15.9	15.4	16.3
Trawbreaga Bay	26-Jul-11	15.2	14.4	16.9	14.9	14.1	16.1
Woodstown Strand	26-Jul-11	14.8	14.4	15.6	14.9	14.1	16.1
Within 30 days of start of mortalities							
Achill Sound	14-Jul-11	17.0	15.6	18.8	19.2	18.0	19.8
Clew Bay	20-Jul-11	15.0	14.2	15.8	15.3	14.0	18.5
Dungloe	03-Jul-11	15.5	15.0	16.2	19.3	18.4	19.9
Lough Swilly	22-Jul-11	20.5	17.4	22.9	14.8	13.7	16.0
Sherkin	11-Aug-11	14.5	13.7	14.9	15.8	14.8	16.5
Trawbreaga Bay	26-Jul-11	15.2	14.3	16.3	15.0	14.0	16.3
Woodstown Strand	26-Jul-11	15.1	14.6	15.7	15.0	14.0	16.3

[a] The bay may also contain other sites.

each of the high mortality sites was also reported, for comparison.

At all high mortality sites with available data, a visual assessment of temperature changes was made during the period when mortality events were first recorded. A low-pass filter using Matlab (MathWorks, Natick, MA, USA), was used to remove the tidal, diurnal and other high frequency signals from the temperature time series, in order to assess the overall trend in temperature during the period when mortality occurred, particularly with respect to a 16 °C threshold.

2.4. Linear mixed model analysis

The cumulative batch-level mortality from placement to first grading was the outcome of interest. The independent variables, for consideration during the risk factor analysis, are presented in Table 1.

Due to the highly skewed nature of the mortality data, the natural logarithm was used to transform these to a normal distribution. In addition the appropriate transformation of the outcome was assessed based on the residuals from the final model, comparing the profile likelihood ratio to identify the optimal box-cox transformation (Dohoo et al., 2009).

A mixed linear regression model of cumulative batch-level mortality was developed using SAS v 9.3 (SAS Institute Inc., 2003). A univariable screening approach was used, where all variables with $p < 0.2$ at the univariable stage became candidates for the multivariable model. The need for a 'farm' random effect was tested within the mixed model, using a likelihood ratio test (Dohoo et al., 2009), to account for clustering of batches within farms. A backward selection procedure was used to eliminate terms from the model based on an F-test ($p > 0.05$). The relationship between continuous predictors and the outcome was examined using plots of the continuous predictors against the logarithm of batch mortality. Where no obvious linear or polynomial relationship was observed, continuous predictors were categorised into four groups based on the corresponding quartiles. Variables that were not significant at the univariable screening stage were added to the final model and tested using an F-test ($p < 0.05$). In addition, variables that, when combined, represent an underlying effect were tested in combination as described by Cohen et al. (2003) by adding the combined variables to the final model. For example, the variables: 'number of times bags were turned' and 'handling at grading' when combined were considered to represent a 'bag handling' effect. The correlation between covariates was evaluated using a chi-square test between nominal variables and kendall's tau-b assessment of correlation between continuous variables. Plots of studentised residuals from the final model and influence plots were examined to identify lack of fit or any outliers from the final model.

3. Results

3.1. Study population

A total of 80 study batches, from 28 farms, located at 24 sites within 17 infected bays were included in the study (Fig. 2). The batches contained between 10 and 4650 bags of oysters with a median number of 215 bags per batch. The median number of oysters per bag as at the end of May 2011 was 2000; this varied between 210 and 15,000 oysters per bag between batches. Cumulative batch mortality ranged from 2% to 100%, with a median batch mortality of 16% and an interquartile range of between 10% and 34% (Figs. 2 and 3).

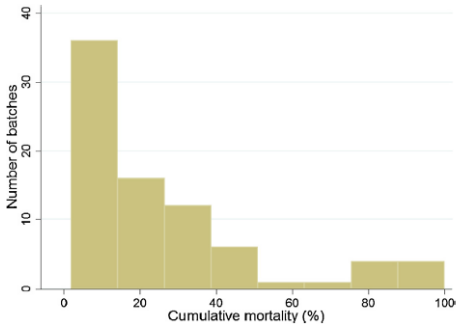

Fig. 3. Distribution of the cumulative mortality (%) among the 80 study batches in Ireland during summer 2011.

3.2. Descriptive analysis of temperature at high mortality sites where start date of mortality recorded

There were 14 high mortality sites (mortality > 34%), with a total of 20 high mortality batches (out of 52 batches at these sites), and 10 low mortality sites (no batches experienced a cumulative batch mortality > 34%), with a total of 28 batches. Of the high mortality sites, 10 sites had a recorded start date for mortalities, however, three of these sites had missing temperature data (three sites in Ballymacoda, Bannow and Lough Foyle bays). The median temperature within 7 and 30 days of the reported start of mortality at the seven high mortality sites with a recorded start date of mortality and relevant temperature data is shown in Table 2. The median temperatures at the 10 low mortality sites during the same time periods as each of the high mortality sites are also presented in Table 2. The interquartile range for the high mortality sites overlapped the interquartile range for the low mortality sites during the 7 and 30 days time intervals, with the exception of the site at Lough Swilly, indicating that there was little difference in temperature between the low and high mortality sites prior to the start of mortalities.

A visual assessment of temperature data during the reported start of mortality at the seven high mortality sites with a recorded start date for mortalities and temperature data prior to the start of mortalities is presented in Appendix B. At four sites (Achill Sound, Dungloe, Trawbreaga Bay and Woodstown Strand), an increase in temperature over 16 °C coincided with the time period when mortality events were first reported; at one site (Sherkin), no visual association was observed; and at two sites (Clew Bay, Lough Swilly), a substantial change in temperature, in this case a decline followed by an increase, was coincident with the time period when mortality was first reported.

3.3. Linear mixed effects regression model

All predictors with a p-value in the univariable analysis of <0.2 were considered in the initial linear mixed effects regression model (Table 3). The risk factors entered into the initial model were: size, ploidy, OsHV-1 µvar bay status, hatchery and the maximum summer temperature. The remaining significant variables after the backward selection process were: OsHV-1 µvar bay status and hatchery. Variables that were not significant at the univariable stage ($p > 0.2$), and combined variables representing an underlying effect were added to the model, however, none were significant. The studentised residuals were plotted against each of the variables not in the current model using a Loess smooth to identify any potential relationships. The Loess plot against 'maximum summer temperature' suggested a possible quadratic relationship. A quadratic term of the 'maximum summer temperature', after centring the variable to account for correlation between the original value and the quadratic term (Dohoo et al., 2009), was significant when added to the model (Table 4). Residuals from the final model indicated no significant lack of fit and no significant outliers. The appropriate transformation of the outcome variable (cumulative batch-level mortality), based on residuals from the initial model (i.e. containing hatchery and OsHV-1 µvar bay status) and comparing the raw mortality, natural logarithm transformation, arcsine transformation and box-cox transformations were assessed. The natural logarithm transformation was deemed as the most appropriate based on residuals and assessment of box-cox transformations and comparing the profile log-likelihood (Dohoo et al., 2009). The 'farm' random effect was significant (likelihood ratio test: $p = 0.023$) and 38% of the variance was between farms and 62% within farms.

A plot of the best unbiased linear estimates of mortality against the 'maximum summer temperature' is shown in Fig. 4, indicating an increase in mortality which appears to level off after a temperature peak was reached. The final model also showed that batches imported from French hatcheries 1, 2, 4 and 5 had significantly lower mortalities than batches imported from Non-French hatchery 2. Non-French hatchery 1 mortalities were not significantly different to those from Non-French hatchery 2. Further, batches grown in bays which tested OsHV-1 µvar negative during the study had significantly lower mortalities than batches grown in bays which had tested positive.

4. Discussion

In this work, we have sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. Increased farmed oyster mortality has been an ongoing concern in Ireland for some years (Malham et al., 2009; Peeler et al., 2012), although there is evidence from the current study of a lower median batch mortality in 2011 (16%) compared with either 2009 (37%) or 2010 (32%). Three risk factors were significantly associated with mortality including the hatchery from which seed was sourced, the presence of OsHV-1 µvar detected in specific bays during 2011 and the maximum temperature observed between June and August 2011, inclusive. Each will be considered in turn.

We noted a strong association between hatchery of origin and mortality, with seed imported from French hatcheries experiencing markedly lower mortality

Table 3
The median cumulative mortality of oyster batches in Ireland in 2011, along with the interquartile range by categorical risk factors that met the criteria for inclusion in the multivariable model ($p < 0.2$).

Risk factor		No. of batches	Cumulative batch mortality			*p*-Value[a]
			Q1	Median	Q3	
Size (mm)	2/3	3	25	39	45	0.083
	4/6	66	9	14	31	
	7+	11	20	34	57	
Ploidy	Diploid	17	10	33	82	0.059
	Triploid	63	8.7	15	32	
OsHV-1 µvar bay status	Negative	13	8	10	12	0.199
	Positive	67	10	20	35	
Hatchery	French hatchery 1	43	9	13	23	0.005
	French hatchery 2	8	9	19	41	
	French hatchery 3	1	25	25	25	
	French hatchery 4	1	7	7	7	
	French hatchery 5	15	8	15	39	
	Non-French hatchery 1	7	12	86	100	
	Non-French hatchery 2	5	34	76	82	

[a] *p*-Value is based on a univariable linear mixed model using log of the final mortality as the outcome variable and 'farm' as a random effect.

compared with seed imported from non-French hatcheries, when placed in bays which were historically infected with OsHV-1 µvar. As illustrated in Table 3, the median cumulative mortality of batches from French hatcheries varied between 7 and 25%, and from non-French hatcheries between 76 and 86%. There are several differences between these two seed stocks, which may at least partly explain this result. The first relates to prior OsHV-1 µvar exposure. This virus is endemic in France (Pernet et al., 2012). Therefore, prior exposure to this virus, either of the seed itself or of the related broodstock, can be assumed. In contrast, the non-French hatcheries are located in areas outside of Ireland which were not previously infected with OsHV-1 µvar. Prior exposure can lead to latency (Dégremont et al., 2013), a common feature of other herpesvirus infections (Arzul et al., 2002), and OsHV-1 µvar has been identified in apparently healthy oysters (Arzul et al., 2002; Barbosa-Solomieu et al., 2004; Dégremont et al., 2013). However, prior exposure will not result in a specific immune response, noting that molluscs lack immunological memory, relying entirely on innate immunity to overcome diseases (Gestal et al., 2008; Renault, 2008). Rather, the protective effect of prior exposure is likely genetic (Dégremont et al., 2007; Sauvage et al., 2009; Huvet et al., 2010), with oysters surviving a mortality event being naturally selected for resistance to disease (Dégremont et al., 2010; Pernet et al., 2012). In recent years, considerable progress has been made in France towards selection for OsHV-1 µvar resistant oysters, particularly in the context of summer mortality in adults (Dégremont et al., 2010, 2013). The second difference between the seed stocks relates to ploidy: a substantial proportion of the French-derived batches in this study were triploid (88%), whereas most of the non-French-derived batches were diploid (75%). In the current study, there was a univariable association between ploidy and cumulative batch mortality (with diploid stock being at greater risk, Table 3), however, ploidy was not retained in the final multivariable model (Table 4). This result is at odds with an earlier Irish study, where triploid oysters were at greater mortality risk (Peeler et al., 2012), but consistent with

Table 4
Linear mixed regression model of the log of batch mortality among 80 batches of oysters in Ireland in 2011.

Variable	Categories	Estimate	Standard error	*p*-Value
Intercept		4.57	0.47	<0.001
Hatchery	French hatchery 1	−1.73	0.46	<0.001
	French hatchery 2	−1.37	0.52	0.010
	French hatchery 3	−1.20	0.84	0.157
	French hatchery 4	−1.97	0.87	0.026
	French hatchery 5	−1.33	0.50	0.011
	Non-French hatchery 1	−0.01	0.53	0.982
	Non-French hatchery 2	0.00	–	–
OsHV-1 µvar bay status	Negative	−0.85	0.37	0.035
	Positive	0.00	–	–
Max. summer temp		0.05	0.03	0.116
Max. summer temp^2		−0.01	0.005	0.040

Random effects	Variance estimate	Standard error
Farm	0.270	0.160
Residual	0.443	0.095

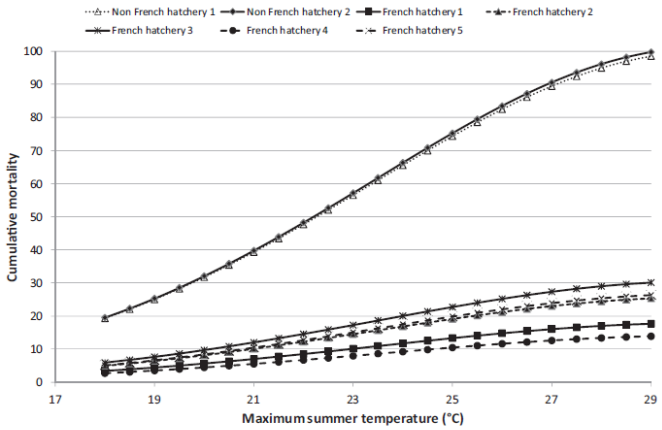

Fig. 4. Predicted best linear unbiased estimator of the cumulative mortality from the multivariable mixed linear model plotted against the maximum summer temperature, for each hatchery in OsHV-1 µvar positive bays.

studies on adults from France, prior to 2006 at least (Gagnaire et al., 2006; Samain, 2011), which found triploid oysters were more resistant to summer mortality. This latter effect varied by season (Pernet et al., 2012), possibly due to seasonal differences in reproductive effort and immunological parameters. Given the data available, it is not possible to disentangle the relative importance of ploidy and prior OsHV-1 µvar exposure in the current study. The association with ploidy could be a consequence of confounding, noting that most of the French seed was triploid and most of the seed imported from non-French hatcheries was diploid. The observed effect could be due to innate resistance among stock from French hatcheries to OsHV-1 µvar infection.

There was also some variation in the mortality of batches from different French hatcheries, with median mortality varying between 7% and 25% (Table 3), although the sample size was too small to conduct a formal analysis as to whether these differences in mortality were significant. In these hatcheries, diploid females are produced locally, whereas the male tetraploids are owned by the French government but moved from one hatchery to another for the purposes of fertilisation. This may reflect a degree of genetic selection in specific hatcheries over the period since 2007/08 when the disease first appeared in France. A genetic component, leading to hatchery differences, has previously been noted for summer mortality in *C. gigas* adult oysters (Dégremont et al., 2007; Sauvage et al., 2009; Huvet et al., 2010).

In this study, the OsHV-1 µvar status of the bay was significantly associated with mortality (median mortality of 20% and 10% among bays where OsHV-1 µvar was detected, or not, during 2011, respectively, Table 3). In this context, bay status is best interpreted in terms of viral load during 2011, as opposed to either presence or absence, noting that we used a sampling strategy to provide 95% confidence that virus would be detected, if present at a specified design prevalence of 10% or greater (Table 1). Each of the 17 study bays can be considered endemically infected: OsHV-1 µvar had been detected in each, either during 2011 or previously, and the virus is known to persist in adult oysters following primary infection (Lipart and Renault, 2002; Dégremont et al., 2013). These observations are consistent with earlier work, highlighting increased mortality risk with increasing quantities of OsHV-1 µvar DNA, but frequently in the context of summer mortality in adults (Pepin et al., 2008; Sauvage et al., 2009; Schikorski et al., 2011; Garcia et al., 2011).

We found a quadratic relationship between the natural logarithm of the cumulative mortality and the maximum temperature observed between June and August 2011, noting that the maximum temperature observed in this study may have occurred as a 1 day event rather than a sustained rise in temperature. In addition, for the seven high mortality sites at which mortality date and temperature data were available, temperature had exceeded 16 °C in the week prior to mortalities at five of the sites. At the other 2 sites, temperatures had either exceeded 16 °C in the previous month (Sherkin) or were around 16 °C in the previous week (Woodstown Strand) (Appendix B). However, there was little difference in the average temperature in the time prior to the mortalities when compared to sites with low mortalities (Table 2). A number of studies have highlighted the multifactorial nature of mortality events in oyster spat and larvae, with the European Food Safety Authority (EFSA, 2010) stating that climatic factors were unlikely to be a sufficient cause for summer mortalities. However, this publication also noted the absence of mortality outbreaks when the water temperature had been below 16 °C. Several authors have highlighted the potential role of increased water temperature in oyster mortality events. During 1998–2006 in France, OsHV-1 was often detected when temperatures increased quickly, but was no longer detected once temperatures were

stable even if they remained high (Garcia et al., 2011). Further, a temperature increase may lead to a re-activation of latent infection (Sauvage et al., 2009). According to data collected from M1–M5 Databuoys deployed around the Irish coast, water temperatures were significantly lower in 2011 than in previous years (Marine Institute, http://www.marine.ie/home/publicationsdata/data/buoys/). Although several authors have highlighted the role of farm management (Samain and McCombie, 2010; European Food Safety Authority, 2010), no relevant variables were significant in the current multivariable modelling.

There are a number of limitations to the current study which we note here. We were reliant on farmer recall to determine the start date of mortalities. This proved problematic, however, both in terms of data validity and completeness. Further, the precision of these estimates is influenced by the frequency with which farmers check their stock. In most cases, due to tidal patterns which lead to site inaccessibility, stock can only be checked at best every two weeks, and in some cases stock may not be checked for up to a month. Similar challenges have been described previously among French oyster farmers, where yearly variation in reporting sensitivity has been observed. Increases in reporting sensitivity were found to occur concurrent with outbreak occurrence and with implementation of financial incentives to encourage farmer reporting of mortality events (Lupo et al., 2012). The cumulative mortality estimate for each batch in the study was based on the outcome of grading for each batch in early spring 2012. Some farmers with several study batches found it difficult to obtain grading data for individual batches as grading would usually be carried out on a stock basis rather than a batch basis. We also faced some gaps in the environmental data, noting that recordings were not taken during the same time period across all sites. In addition, although data were available on a range of environmental parameters, only the temperature data proved suitable for subsequent analysis. Extra environmental data were only recorded at a limited number of sites due to cost and availability of monitoring equipment. There were some issues with tracking batches once they had been split. Several batches were split during the season, resulting in new stocking densities with new bag mesh sizes, and several new locations for the split batch. This proved problematic when tracking split batches through the season, as once the original batch was split, all subsequent batches had a separate set of data, and were essentially a new batch. Initial sample size calculations were based on the difference in the proportion of mortality in two groups. However, for the analysis linear regression with cumulative mortality as the outcome was used. If sample size calculations had been based on the difference between two mortality rates as described by Lwanga and Lemeshow (1991) there would have been very little difference in the estimated sample size. Finally, the farms and study batches were not chosen at random, primarily for logistical reasons. It is possible that these samples are not representative of the broader oyster farming population in Ireland.

The study provides some insights into mortality events affecting farmed Pacific oyster production in Ireland. Batch mortality was lower in 2011, compared with earlier years, in association with lower viral loads. Mortality was significantly associated with hatchery source, for reasons that are currently unclear, and with water temperature. Further work is needed to elucidate the basis for this effect. The relative importance of prior OsHV-1 µvar infection and ploidy will become clearer with ongoing selection towards OsHV-1 µvar resistant oysters. Work is currently underway in Ireland to investigate these factors further, by tracking seed from various hatchery sources which were put to sea in 2012 under similar husbandry and environmental conditions.

Conflict of interest

The authors do not have any conflict of interest.

Acknowledgements

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Appendices A and B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2013.10.023.

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Appendix D: Poster presentation for the "16th International Conference on Diseases of Fish and Shellfish" to be held in Tampere (Finland), September 2013

An Epidemiological Study of the effect of OsHV1-µvar on *C. gigas* seed in 2011

Tracy A. Clegg, Teresa Morrissey, Fiona Geoghegan, S. Wayne Martin, Kieran Lyons, Seán Ashe, Simon J. More

Introduction

The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland. Episodes of increased mortality in *C. gigas* have been described in many countries, and in Ireland since 2008. The cause of mortality events in *C. gigas* spat and larvae is suspected to be multifactorial, with Ostreid herpesvirus 1 (OsHV-1, in particular OsHV-1 µvar) considered a necessary, but not sufficient, cause.

The objectives of the current study were to describe mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and oyster endogenous factors. A prospective cohort study was conducted during 2010 to 2012, involving 80 study batches, located at 24 sites within 17 bays.

All study farmers were initially surveyed to gather relevant data on each study batch, which was then tracked from placement in the bay to first grading. The outcome of interest was cumulative batch-level mortality (%). Environmental data at high and low mortality sites were compared.

Materials and methods

The study aimed to track 80 batches of *C.gigas* in 17 bays around the coastline (Figure 1)

A batch of oysters was the unit of interest and was defined as a variable number of oysters of similar size, originating from one hatchery, placed at a particular site within a bay at one point in time.

The study period was from the time of first batch immersion (5th May 2010) through to the date of last batch graded in spring/summer 2012 (12th April 2012).

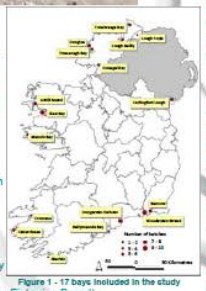

Figure 1 - 17 bays included in the study

An initial survey of all participating farmers was conducted to gather relevant data on each study batch. The survey was administered in a joint effort by Bord Iascaigh Mhara (BIM; the Irish Sea Fisheries Board) Regional Officers and the Fish Health Unit of the Marine Institute (MI) to collect data on batch identification, risk factors of interest, batch mortality data, splitting, grading and handling frequency. For any batch where mortality occurred, details of the mortality event were recorded (i.e. start and finish date of the mortality episode, estimated percentage mortality in the batch, predisposing factors etc), and samples of the stock were taken for laboratory analysis where possible and tested for the presence of OsHV-1 µvar by PCR analysis (Pepin et al; 2008)

A total of 55 data loggers (Figure 2), at least one per bay, were deployed to obtain environmental data. These were deployed in May 2011 on a number of sites, with further deployments made in early June 2011 on the remaining sites. A data logger was placed on each participating site. Previous studies found that increase in temperature was a risk factor that should be investigated. It has been noted that no outbreaks had been reported when the water temperature was below 16°C (EFSA, 2010).

Figure 2 – Data loggers deployed in eaoh of the 17 study bays

All the data was submitted to Centre for Veterinary Epidemiology and Risk Analysis (CVERA) in UCD (University College Dublin) for risk analysis on all variables. Descriptive analysis was carried out on temperature data when time of mortality was recorded, which included a visual assessment of temperature data to assess whether the 16°C threshold had been a significant factor. Linear mixed model analysis was carried out on all variables, with the cumulative batch-level mortality from placement to first grading was the outcome of interest.

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Results

This epidemiology study sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. The main findings of the study are:

Lower average mortality levels compared to previous years (2009-2010)
- Average batch mortality in 2011 - 16% (37% 2009, 32% 2010)

Hatchery Source
- French hatchery seed lower mortality than Non-French hatchery seed when placed in OsHV-1 µvar positive bays in Ireland

OsHV-1 µvar viral load
- OsHv-1 µvar detection significantly associated with mortality in *C. gigas* oysters

Other factors
- Range of other factors considered – Farm Management risk factors were found not significant in this study
- Temperature – an increase in mortality which appears to decrease/level off at after a temperature peak had been reached

A total of 80 study batches, from 28 farms, were located at 24 sites within 17 OsHV-1 µvar infected bays. The batches contained between 10 and 4,650 bags of oysters with a median number of 215 bags per batch. The median number of oysters per bag as at the end of May 2011 was 2,000; this varied between 210 to 15,000 oysters per bag between batches. Cumulative batch mortality ranged from 2% to 100%, with a median batch mortality of 16% and an interquartile range of between 10% and 34% (Figure 3).

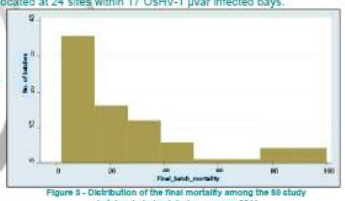

Figure 3 - Distribution of the final mortality among the 80 study batches in Ireland during summer 2011

There were 14 high mortality sites (mortality >34%), with a total of 20 batches, and 10 low mortality sites (no batches experienced a cumulative batch mortality > 34%). Of these 14 high mortality sites, 10 sites had a recorded start date for mortalities, however, three of these sites had missing temperature data (three sites in Ballymacoda, Bannow and Lough Foyle bays). The median temperature within 7 and 30 days of the reported start of mortality at the seven high mortality sites with a recorded start date of mortality and relevant temperature data.

Conclusions

We sought to identify risk factors associated with increased mortality of farmed Pacific oysters in Ireland during 2011. The current study showed evidence of a lower median batch mortality in 2011 (16%) compared with either 2009 (37%) or 2010 (32%). Three risk factors were significantly associated with mortality:

Hatchery Source

There was a strong association between hatchery origin and mortality, with French hatchery seed having significantly lower mortality than Non-French seed, when placed in OsHV-1 µvar positive bays in Ireland. Differences were also evident between hatchery stock, in relation to OsHV-1 µvar exposure – Non-French stock is naïve to OsHV-1 µvar and French stock is pre-exposed to the virus, and it has been shown that prior exposure may lead to a degree of immunity (European Food Safety Authority, 2010, Arzul et al., 2002). There was a significant association between ploidy and batch mortality with diploid at greater risk, however, this finding is compromised by the fact that 88% of French stock was triploid and 75% Non-French stock was diploid. There was also some variation in the mortality of batches from different French hatcheries.

OsHV-1 µvar viral load

The viral load of OsHV-1 µvar in each participating bay was assessed during the study and OsHV-1 µvar status was found to be significantly associated with mortality. This finding is consistent with previous work, where it has been shown that there is an increased mortality risk with increasing quantities of OsHV-1 µvar DNA (Sauvage *et al.*, 2009; Schikorski *et al.*, 2010).

Temperature exceeding 16°C between June and August 2011

A quadratic relationship between the natural logarithm of the cumulative mortality and the maximum temperature observed between June and August 2011, noting that the maximum temperature observed in this study may have occurred as a one day event rather than a sustained rise in temperature. For the seven high mortality sites at which mortality date and temperature data were available, temperature had exceeded 16°C in the week prior to mortalities at five of the sites. At the other 2 sites, temperatures had either exceeded 16°C in the previous month (Sherkin) or were around 16°C in the previous week (Woodstown Strand). However, there was little difference in the average temperature in the time prior to the mortalities when compared to sites with low mortalities.

Other Factors

A range of other factors were analysed such as; Time in water, Bag mesh size, Age placed in water , Journey duration, Handling at grading, Position on shore, Bay, Sea bed type, Bag split. All of these farm management risk factors were analysed but were found to be not significant in this study.

Acknowledgments

We gratefully acknowledge the assistance of participating oyster farmers, data collection by Bord Iascaigh Mhara inspectors and Marine Institute staff and assistance from Dan Collins (UCD Centre for Veterinary Epidemiology and Risk Analysis) with mapping.